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TOXICOLOGY DETACHMENT

CHARACTERIZATION OF THE METABOLISM, DISTRIBUTION AND TOXICITY OF 2,6-di-*t*-BUTYL-4-NITROPHENOL FOR PURPOSES OF HEALTH HAZARD ASSESSMENT

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FOR THE COMMANDING OFFICER

KENNETH R. STILL, CAPT, MSC, USN
Officer-in-Charge
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In 1992, the Navy Environmental Health Center, Norfolk, VA was made aware of the concern about the discoloration (yellowing) of interiors (e.g. bulkheads and bedding) and the possible exposure of navy personnel aboard submarines, to an unknown substance. The agent was identified as 2,6-di-tert-butyl-4-nitrophenol (DBNP). The yellowing process appeared to arise from the reaction of 2,6-di-tert-butylphenol (DBP), an antioxidant additive used in engine lubricant, with NO2 in the submarine atmosphere. A research program was initiated for health hazard assessment of DBNP. This technical report summarizes the results of our research program and the information available in the literature. The LD-50 dose by intraperitoneal route in rodents is above 250 mg/Kg. DBNP is half as toxic by oral route and there is no sign of toxicity or skin irritation at high doses applied dermally. Even though these acute toxicity tests demonstrate that DBNP has a low toxicity, tissue distribution, metabolism and excretion studies carried out in rats clearly suggests, that DBNP has considerable tendency to produce cumulative toxic effects at low doses due to slow excretion and storage in fats. At the cellular level the toxicity expressed by DBNP is very likely due to its inhibitory effects on ATP synthesis.

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PREFACE

This is one of a series of technical reports describing results of the experimental laboratory programs conducted at the Naval Medical Research Institute Detachment (Toxicology). This document serves as report on the Characterization of the Metabolism, Distribution and Toxicity of 2,6-di-*t*-BUTYL-4-Nitrophenol for Purposes of Health Hazard Assessment. The research described in this report began in October 1993 and was completed in April 1995 under Navy Contract No. M0096.004.1405. This study was sponsored by the U.S. Navy under the direction of CAPT Kenneth R. Still, MSC, USN.

The opinions contained herein are those of the authors and are not to be construed as official or reflecting the view of the Department of the Navy or the Naval Services at large.

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PREFACE

This is the first technical report that summarizes the findings of the research project entitled "Characterization of the metabolism, distribution and toxicity of 2,6-di-*t*-butyl-4- nitrophenol (DBNP) for purposes of Health Hazard Assessment." The research described in this technical report began in October, 1993 and was completed in April, 1995. This study was sponsored by the U.S. Navy under the direction of the Officer-in-Charge (OIC) of the Toxicology Detachment, Naval Medical Research Institute, CAPT David A. Macys, MSC, USN (the initiating OIC) and CAPT Kenneth R. Still, MSC, USN (the terminating OIC) and was supported by the Naval Medical Research and Development Command, Task No: 63706- M0096-004-1405. The scientific objective of this study was to gain toxicological information about DBNP. The research described in this technical report was carried out at the Naval Medical Research Institute Detachment (Toxicology) Bldg. 433 Area-B, Wright-Patterson Air Force Base, OH 45433-7903 by Navy, civil service, and contract scientists under the scientific supervision of CDR John Wyman, Ph.D., MSC, USN (Principle Investigator), and, upon his retirement from the Navy, Dr. Robert L. Carpenter, Ph.D. (Senior Scientist/Principle Investigator). The assistance of the following individuals during various phases of this research effort are gratefully acknowledged: J.A. Rivera, MS, for his analytical chemistry evaluation of DBNP samples and his development of a synthesis procedure for DBNP; HM1 C. Alva, USN; and HM3 D.L. Lee, USN for their untiring assistance in the laboratory during the rat liver perfusion experiments.

The animals used in this study were handled in accordance with the principles stated in the *Guide for the Care and Use of Laboratory Animals*, prepared by the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council, Department of Health and Human Services, National Institute of Health Publication # 86-23, 1985, and the Animal Welfare Act of 1966 as amended.

The opinions expressed herein are those of the authors and are not to be construed as official or reflecting the views of the Department of the Navy or the Naval Service at large.

LIST OF TABLES & FIGURES

ABBREVIATIONS

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
C-18	Carbon-18
DBNP	2,6-di-t-butyl nitrophenol
DBP	2,6-di-t-butylphenol
DMSO	Dimethyl sulphoxide
DPM	Disintegration Per Minute
DSC	Differential Scanning Calorimetry
HPLC	High Performance Liquid Chromatography
i.p.	Intraperitoneal
IR	Infra Red
i.v.	Intravenously
LD-50	Lethal Dose-50
NMR	Nuclear Magnetic Resonance
NO_2	Nitrogen Dioxide
RP	Reverse Phase
TCA	Trichloroacetic Acid
TGA	Thermal Gravimetric Analysis

ABSTRACT

In 1992, the Navy Environmental Health Center, Norfolk, VA was made aware of the concern about the discoloration (yellowing) of interiors (e.g. bulkheads and bedding) and the possible exposure of Navy personnel aboard submarines, to an unknown substance. The agent was identified as 2,6-di-tert-butyl-4-nitrophenol (DBNP). The yellowing process appeared to arise from the reaction of 2,6-di-tert-butylphenol (DBP), an antioxidant additive used in engine lubricant, with NO_2 in the submarine atmosphere. A research program was initiated for health hazard assessment of DBNP. This technical report summarizes the results of our research program and the information available in the literature. The LD-50 dose by intraperitoneal route in rodents is above 250 mg/Kg. DBNP is as half toxic by oral route and no sign of toxicity or skin irritation at high doses applied dermally. Even though these acute toxicity tests demonstrated that DBNP has a low toxicity, tissue distribution, metabolism and excretion studies carried out in rats clearly suggests, that DBNP has considerable tendency to produce cumulative toxic effects at low doses due to slow excretion and storage in fats. At the cellular level the toxicity expressed by DBNP is very likely due to its inhibitory effects on ATP synthesis.

SECTION 1

INTRODUCTION

BACKGROUND

This technical report is the final report summarizing the research activities executed by the Naval Medical Research Institute Detachment Toxicology (NMRI/TD) under work unit NMRDC Task No. 63706-M0096-1405 and serves as a final activity report for that work unit. The scope of research is the result of a collaborative effort to address rapidly the emerging submarine fleet toxicity issues involving NMRI/TD, the Navy Environmental Health Center (NEHC) and NAVSEA engineering personnel (Code 390).

This work unit was originally conceived to develop advanced methods of detecting liver toxicity resulting from exposure to toxic chemicals. However, in March of 1992, NEHC received inquiries concerning a yellow discoloration of bulkheads aboard submarines which occurred while they were underway. Subsequent investigation suggested that the causative agent was 2,6-di-*t*-butyl-4-nitrophenol (DBNP) produced as a result of nitration of a phenolic additive to engine lube oil (2,6-di-*t*-butylphenol, DBP) which was presumably released into the air during operations. The findings leading to this hypothesis are reviewed in Appendix A. NEHC contacted NMRI/TD with a request for toxicity data on DBNP. Review of the toxicology literature (see below) lead to the conclusion that additional toxicity study was warranted, and DBNP was chosen as the test agent for this work unit. Work unit goals were modified to include toxicity evaluation of DBNP.

EXISTING TOXICOLOGY DATA

DBNP was originally evaluated for use as a mitocide but was found to have low acute toxicity to mammals (Vesselinovitch *et al.* 1961). Acute toxicity studies carried out in rats,guinea pigs and mice demonstrated that this compound has low toxicity (i.p. LD_{50} 270 mg/kg for rats; 580

mg/kg for guinea pigs, and 700 mg/kg for mice). Daily administration exceeding one twenty-fifth of the LD_{50} dose in rats produced 40% mortality. Holder *et al.* (1971) reported that after i.p. injection, DBNP is excreted in the urine, feces and a small amount in the bile. Orally administered DBNP was poorly absorbed from the gut (30% excreted unchanged) but, once absorbed, was excreted as a glucuronide conjugate. No other metabolites were detected.

NMRI/TD EVALUATION

Apart from the two papers published be Vesselinovitch *et al.* and Holder *et al.*, there is little information concerning the toxicity and mechanism of action of DBNP. This study was undertaken to evaluate the toxicity of DBNP and, if possible, provide clinical parameters that could be routinely monitored as part of medical surveillance of DBNP-exposed submariners. The present investigation was undertaken to study the metabolism, tissue distribution and tissue specific toxicity of DBNP in Fischer-344 rats. Metabolism and tissue distribution studies were carried out using ^{14}C-DBNP (ring-labeled). Toxicity studies were carried out both *in-vitro* and *in-vivo* using tissue slices and a hepatocyte cell line in culture. Biliary excretion studies were performed using a rat liver perfusion procedure. This report summarizes the work carried out in our laboratories and that from the literature.

SECTION 2

MATERIALS AND METHODS

ANIMALS

Upon receipt from the Charles River Breeding Labs (Raleigh, NC) male and female Fischer-344 (F-344) rats, weighing 200-250g, were quality control tested prior to use in the studies. Rats were individually housed in stainless steel wire-mesh cages with water and feed (Purina Rat chow # 5008) available *ad libitum*. The vivarium in which the animals were housed is maintained at 21 to 25° C with 12-h light/dark cycle (light cycle start at 0700 hours). Once the control (vehicle alone) and experimental (DBNP) groups were treated, they were transferred to individual plastic (Nalgene) metabolic cages. All rats were identified by tail tattoo. Scientists involved in the handling of animals underwent a yearly animal handler's medical check-up as well as attended the video tape demonstration and lecture on the care and use of laboratory animals mandated by the Animal Care and Use Committee.

CHEMICALS

All the chemicals used in this study are of analytical grade and the solvents are of HPLC grade. They were purchased from the Sigma Chemical Co. (St. Louis, MO) or from the Fisher Scientific Co. (Pittsburgh, PA). Radioactive ^{14}C-DBNP (ring labeled) was custom ordered from the Sigma Chemical Co.

Handling of radioactive chemicals and the disposal of radioactive wastes were in strict accordance with NRC guidelines governed by the Wright-Patterson Air Force Base radiation safety officer.

DBNP SYNTHESIS

A clear pale yellow solution of 2,6-di-*t*-butyl phenol (DBP Fig.1; Sigma Chemical Co.) was prepared in hexane (106g of DBP in 400 ml of hexane) with gentle stirring. A fritted glass tube was connected to an NO_2 gas cylinder (99.5% pure; Matheson Gas Company) using polypropylene tubing, and NO_2 was bubbled through the stirred solution at the rate of 500 ml/minute at ambient temperatures for an hour. Beige crystals began to appear approximately 20 minutes after bubbling of NO_2 began and the precipitation was completed within 1 hour. The solid was filtered and washed three times with 50 ml of hexane. The washed crystals were placed in (400 ml) boiling hexane (69° C) and sonicated for 10 minutes to enhance dissolution. Fine needles recrystalized after cooling the solution to room temperature. The crystals were filtered from the mother liquor and vacuum dried. Physical and spectral characteristics of the purified DBNP crystals were determined by doing: elemental analysis; X-ray crystallography; Thermal Gravimetric Analysis (TGA); Differential Scanning Calorimetry (DSC); GC-MS spectrophotometry; UV-VIS spectrophotometry; Fourier-Transformed ^{1}H-NMR ; Fourier - Transformed ^{13}C-NMR ; and Fourier-Transformed IR spectrophotometry (J.A. Rivera-Nevares *et. al.* 1995).

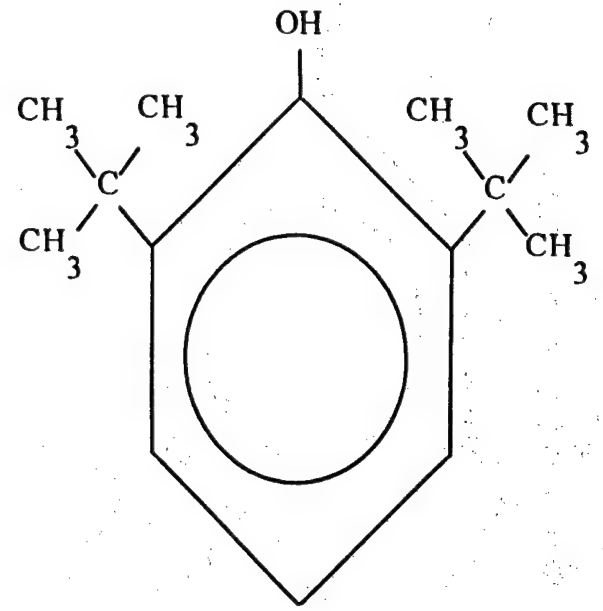
TOXICITY STUDIES

Cold DBNP stock: 10 mg/ml in 80% DMSO, pH adjusted to 7.4 with 0.5% $NaHCO_3$

Radioactive (^{14}C)DBNP stock: 0.456 mg/10 μCi/ml in 80% DMSO pH adjusted to 7.4 with 0.5% $NaHCO_3$.

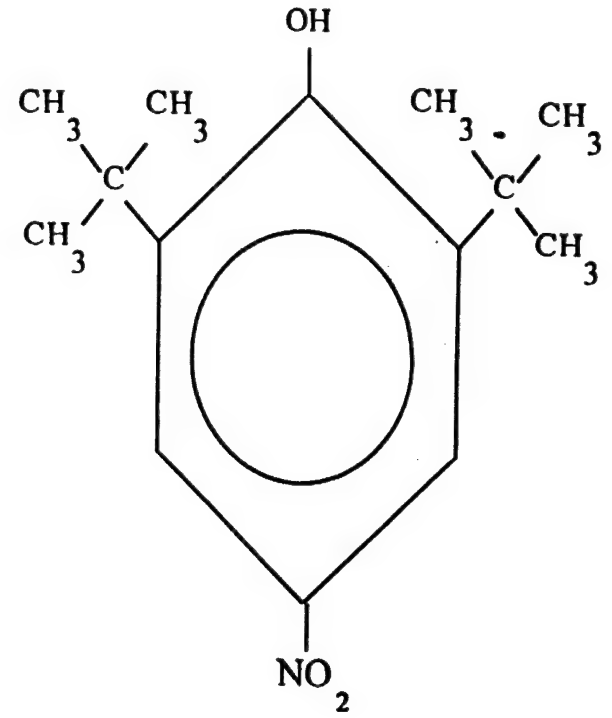
Rats (4 male) were administered with cold DBNP i.p. (10 mg/kg dose) for 10 days. The control group (4 male) received the vehicle alone (80% DMSO with pH adjusted to 7.4 with 0.5% $NaHCO_3$). Both the control and experimental groups were monitored for water consumption, urine and feces production and body weight for 10 days. All of the rats were closely monitored for abnormal changes in behavior.

Figure 1.



2, 6-bis (1,1-dimethylethyl) Phenol

Figure 2.



2, 6 di-t-butyl-4-nitrophenol

CLEARANCE OF DBNP FROM THE BLOOD

The rate of clearance of ^{14}C-DBNP from the blood was measured in male F-344 rats (200-250 g) following administration of 2μ Ci of DBNP (200 μl volume and 91.2 μg of DBNP/rat by i.p., i.v. [tail vein], and oral routes). After the administration of DBNP, a small sterile cut was made in the mid portion of the tail with the surgical blade, and 20 μl of blood was collected at each time point. Each sample was counted in a PACKARD liquid scintillation counter with 10 ml of cocktail (Scintiverse-Fisher Scientific Co. Pittsburg, PA). Quenching corrections were made, and the amount of radioactivity in the sample was expressed in DPMs. At the start of the experiment, blood was withdrawn from the tail cut at 10-minute intervals for an hour and was followed by 15-minute intervals for the next hour. During the third and fourth hours, samples were drawn at 30-minute intervals. From fifth hour onward, samples were taken hourly. At the end of the experiment, the rats were sacrificed, and the amount of radioactivity in different tissues was quantitated (see details below).Three rats were used for each route of administration.

EXCRETION OF DBNP IN THE URINE AND FECES

Rats were administered with ^{14}C-DBNP (2μCi/rat i.p.) and transferred to plastic metabolic cages for the collection of the urine and feces. Feces and urine were collected every 24 hours for 10 days. The urine volume was measured and aliquots (500 μl) were counted for radioactivity. The feces were weighed and homogenized with cold normal saline (10ml/g of feces) using a Polytron-tissue homogenizer(Brinkmann Westbury, NY) An aliquot (1ml) was counted for radioactivity.

TISSUE DISTRIBUTION OF DBNP

Rats were administered with ^{14}C-DBNP (2 μCi/rat i.p.) and sacrificed after 24 hours. The liver, kidney, spleen, heart, brain and fat were dissected, weighed, and homogenized with cold normal saline (10ml/g of tissue) and an aliquot was taken for counting.

ISOLATION, PURIFICATION AND IDENTIFICATION OF DBNP METABOLITE(S) FROM THE URINE AND FECES

Three rats were administered with ^{14}C-DBNP (2μCi / rat i.p) and the urine and feces from each rat was collected and pooled. The urine and feces samples were processed separately. The pooled urine was centrifuged at 10,000 x g for 20 minutes in a Sorval RC-5B, and the supernatant was collected and lyophilized. The lyophilized material was dissolved in a minimal amount of water and treated with powdered activated charcoal (100mg/10 ml) overnight with gentle stirring at 4° C. After charcoal treatment, the samples were centrifuged at 20,000 x g for 30 minutes. The pellet was washed with water (three to four times) until there was no radioactivity present in the supernatant. The supernatants from each centrifugation were pooled and lyophilized. The lyophilized material was dissolved with a minimal amount of water and loaded onto three C-18 bond-Pak columns arranged serially. The C-18 bond pack column was primed with methanol followed by water washing before the samples were loaded. After loading the samples, the column was washed extensively with water, and all of the eluent was pooled and lyophilized. The lyophilized material was dissolved in a minimal amount of water and applied to an HPLC column.

The pooled feces were homogenized with water (10ml/g of feces) using a Polytron homogenizer and centrifuged at 10,000 x g for 20 minutes in a Sorval RC-5B centrifuge. The supernatant was saved, and the pellet was washed three times with water followed by centrifugation. All of the supernatants were pooled and lyophilized. Further processing of the feces samples was done by the same procedures as described for the urine.

Subsequent purification of the sample was carried out using a Beckman HPLC Gold System. Separation of the DBNP and its metabolite(s) was achieved using a C-18 reverse phase column (LiCheosphere 100 RP-18 endcapped (5 μm); Column length -250 mm; Internal Diameter- 4 mm Hewlett-Packard, USA). The separating column was protected with a C-18 guard column. Samples were loaded onto the column (55min/run) in an aqueous phase and maintained in that phase for 5 minutes using 100 % water, and a linear gradient of methanol from 0 to 100 % was achieved in 15 minutes. The column was maintained in 100% methanol for 5 minutes and reverted back to 100 % water at the end of the run.. The peaks were detected with an on-line UV-VIS spectrophotometer and by a radioisotope detector. The radioactive peaks with the same retention times were pooled, concentrated, and rerun on the HPLC with the same eluting conditions to check the purity. In each purification step, a small aliquot was counted to calculate the percentage of recovery.

IN-VITRO LIVER PERFUSION

Isolated perfused liver was prepared according to the method of Miller *et. al.* (1951) with minor modifications. All the surgical procedures were carried out under aseptic conditions. The rat was anesthetized with ether, and the liver was exposed. The common bile duct was cannulated with PE-10 tubing. The animal was heparinized with 1000 units of sodium heparin injected through the inferior vena cava. Immediately after the injection, the inferior vena cava was ligated anterior to the site of injection. The portal vein was cannulated with PE-240 filled with the perfusate. The outflow cannula was inserted into the right atrium, and the liver was removed with the diaphragm and immediately placed in a container of warm saline. This entire setup was placed in a humidified atmosphere. The liver was perfused with Krebs-Henseleit-Ringer [(NaCl-(118 mM); KCl-(4.7 mM); $CaCl\cdot 2H_2O$-(10 mM); $MgSO_4 7\ H_2O$;(1.2 mM); KH_2PO_4 -(1.2 mM); $NaHCO_3$ -(25mM); dextrose- (11.5 mM); pH 7.4] at the rate of 25ml/minute at 37°C with 95-5% O_2-CO_2 . The perfusate was supplemented with 5 mM sodium taurocholate to maintain the bile flow throughout the experiment. After an equilibration time of 30 minutes,

2μCi of ^{14}C-DBNP was added to the perfusion fluid, and the bile was collected. The effluent from the outflow cannula was reefed to the reservoir. The perfusion of the liver with DBNP in the closed system was carried out for 2 hours. The viability of the liver was monitored during the perfusion period by measuring the oxygen consumption. The oxygen consumption by the liver was calculated by measuring the oxygen tension in the perfusate before it enters the liver and after it effuses from the liver. This was repeated four times, and the bile was pooled and analyzed for the metabolite(s).

EFFECT OF DBNP ON HUMAN AND RAT LIVER SLICES

This study was carried out by VITRON, INC. Tucson,AZ 85747. Human liver tissue was obtained from the Association of Human Tissue Users (Tucson, AZ). The liver was procured for transplantation by organ banks but was not used for transplantation for various medical reasons. Once it was decided that the tissue would not be suitable for transplantation, it was immediately placed in an ice-cold solution of Viaspan (a cold preservation solution from DuPont Pharmaceuticals [Wilmington, DE]). Tissue slices were prepared both from human and rat tissue using a Brendal/Vitron tissue slicer and kept in V-7 preservative medium (Tucson, AZ). The slices (200μm) in V-7 preservation medium were floated onto Teflon®/vitron/titanium rollers. The rollers were then carefully blotted and loaded horizontally into glass scintillation vials containing 1.7 ml of Waymouth's culture medium which had been supplemented with 10% fetal calf serum (Hyclone Laboratories), 10 ml/l Fungi-Bact, 84μg/ml of gentamicin, 3.5 mg/ml L-glutamine, and 2.4g/l sodium bicarbonate. Vials were closed with a cap which had a central hole of approximately 2mm, placed in the dynamic organ culture incubator and gassed with 95%-5% O_2/CO_2 mixture. The liver slices were acclimatized for period of 1 hour prior to exposure to individual nitrophenols. Nitrophenols, in an amount required for a specific concentration, were dissolved in 100 μl of DMSO and added to the incubation medium. Control exposures contained 100μl of DMSO alone in the medium. Measurement of intracellular K^+, protein synthesis, LDH leakage and ATP content were used as markers of toxicity.

Intracellular K^+ content

At the end of the incubation period, the slices were removed and homogenized in 20 µl of cold 70% perchloric acid followed by centrifugation. The supernatant was assayed for K^+ using a Model CA-51, Perkin-Elmer Flame Photometer. Results are expressed as µmol of K^+/g wet weight.

Protein Synthesis

Slices were exposed to ^3H- leucine for a specific period of time. At the end of the exposure, the slices were removed and washed three times with the buffer. The washed slices were homogenized with 15% ice-cold TCA followed by centrifugation. The precipitated protein was washed three times with ice-cold TCA and counted in a liquid scintillation counter. Results were expressed as DPM/mg wet weight.

LDH Leakage

Lactate dehydrogenase activity in the incubation medium was measured using an LDH assay kit from Sigma Chemical Co.

ATP Content

ATP content was measured in the TCA homogenate using a luciferin-luciferase assay kit purchased from Sigma Chemical Co.. Results are expressed as nmol ATP/mg wet weight.

EFFECT OF DBNP ON THE HEPATOCYTE CELL LINE

WP-344 cells are routinely maintained in Gibco MEM medium supplemented with 10% fetal calf serum from Hyclone Laboratories. Confluent cells in 100 mm plates were washed once

with Gibco MEM medium (no serum) and exposed to DBNP (0.1μg-2μg/ml) for 16 hours. The control cells were exposed to 5μl of 80% DMSO (adjusted to pH 7.4 with 0.5% $NaHCO_3$). At the end of the incubation period, the cells were counted for viability by the tryphan blue dye exclusion method with hemocytometer counting.

MITOCHONDRIAL RESPIRATION

Mitochondria were prepared from rat liver by the following method. Rats were decapitated, and the liver was quickly removed and placed in an ice-cold 0.25M sucrose in 0.07M Tris-HCl buffer pH 7.4 (10ml buffer/g tissue). After the liver was minced into small pieces, it was homogenized using a Potter-Elveign homogenizer (Brinkmann, Westbury, NY). The homogenate was centrifuged at 2500 x g for 10 minutes. The supernatant was saved and centrifuged at 10,000 x g for 10 minutes. The crude mitochondrial pellet was washed three times with Tris-HCl sucrose buffer.

Isolated mitochondria were placed into an 1-3 ml oxygen electrode cell with a stirrer containing the reaction mixture. The mixture was composed of 40 mM Tris-HCl pH 7.5; 5 mM K_2HPO_4; 5 mM Mg SO_4 and 100 mM KCl with 2 mg of mitochondrial protein/ml.

A stable recorder baseline was obtained before the initiation of state-4 respiration by adding succinate to yield a final concentration of 5 mM. After 1-2 minutes of state-4 respiration, state-3 respiration was initiated by adding 5μ moles of ADP. The effect of DBNP on state-4 and state-3 mitochondrial respiration was compared with a known uncoupler of mitochondrial oxidative phosphorylation, DBP.

EFFECT OF DBNP ON RAT LIVER FATTY ACID BINDING PROTEIN AND RAT LIVER SULFOTRANSFERASES

This study was carried out by Dr. Sanford S. Singer Ph.D at University of Dayton, Dayton ,OH. Fatty acid binding protein (FASB) and sulfotransferases which includes bile acid

sulfotransferase (BST), dopamine sulfotransferase (DST), cortisol sulfotransferase (HCST) and estrogen sulfotransferase, were prepared from rat liver cytosol using the standard procedure. The effect of DBNP (200 μ M) on FABP protein was measured *in-vitro* using rose bengal assay method.. Sulfotransferases (BST, DST, HCST and EST) were routinely assayed using the corresponding substrate (bileacid, dopamine, cortisol and estrogen) in the assay mixture containing radioactive coenzyme 3' - phosphoadenosine-5'-phosphosulfate (PAPS- ^{35}S). The effect of DBNP (200 μ M) was measured on all four sulfotransferases. FABP and sulfotransferases activity were also determined in rat liver perfused with DBNP(0.36 mM) and also in liver of rats administered with DBNP (25mg/kg, i.p. for 60 days).

SECTION 3

RESULTS AND DISCUSSIONS

The reaction conditions for the synthesis DBNP from DBP are shown in Fig .3. Under these conditions, the yield was 75%. The elemental analysis of the purified product showed the chemical composition as being 67.09% carbon, 8.16% hydrogen, and 5.49% nitrogen. The theoretical values for DBNP are 66.95% carbon, 8.36% hydrogen, and 5.58% nitrogen. HPLC analysis of the purified product by Sigma Chemical Co. indicated a purity of 99.5%.

The single crystal structure of DBNP grown in hexane is shown in Fig.4. DBNP crystallizes in the acentric orthorhombic space group $Pna2_1$. The nitro group is virtually coplanar with the aromatic ring and exhibits an O2-N-C4-C3 torsional angle of 0.4(6)° . The hydroxy- group hydrogen H1 is rotated from the plane of the aromatic ring to exhibit the C6-C1-O1-H1 torsional angle of -17(4)° . An intermolecular hydrogen-bonding interaction was found between the hydroxy-group hydrogen H1 of one DBNP molecule and nitro-group oxygen O2 of an adjacent one in an head-to-tail arrangement.

The Thermal Gravimetric Analysis (TGA) shows that DBNP begins to lose its mass at 125°C and achieves its fastest rate of mass loss at 177.83°C. It lost 100% of its original mass at about 212°C.

The DSC thermogram analysis of DBNP showed two endotherms. One occurred at the extrapolated onset of 157.8°C (ΔH_1 = 123.5 J/g) and the other, after cooling and reheating, at the extrapolated onset of 152.49°C (ΔH_2=85 J/g). Thus, the data suggest that two well-defined crystalline domains exist for DBNP . The proposed existence of two stable packing configurations is further supported by the typically encountered discrepancy in the melting point profile reported for DBNP (152 to 153°C *vs* 156 to 157 °C).

The physico-chemical properties of DBNP are: a yellow powder; a melting point of 157°C;

Figure 3. Synthesis of DBNP

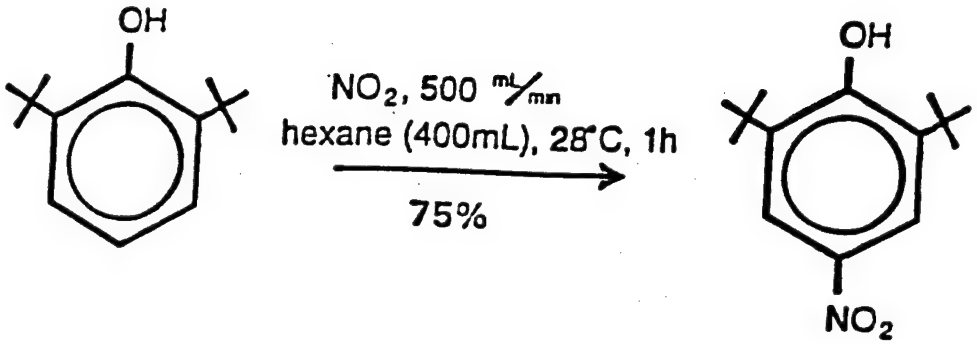
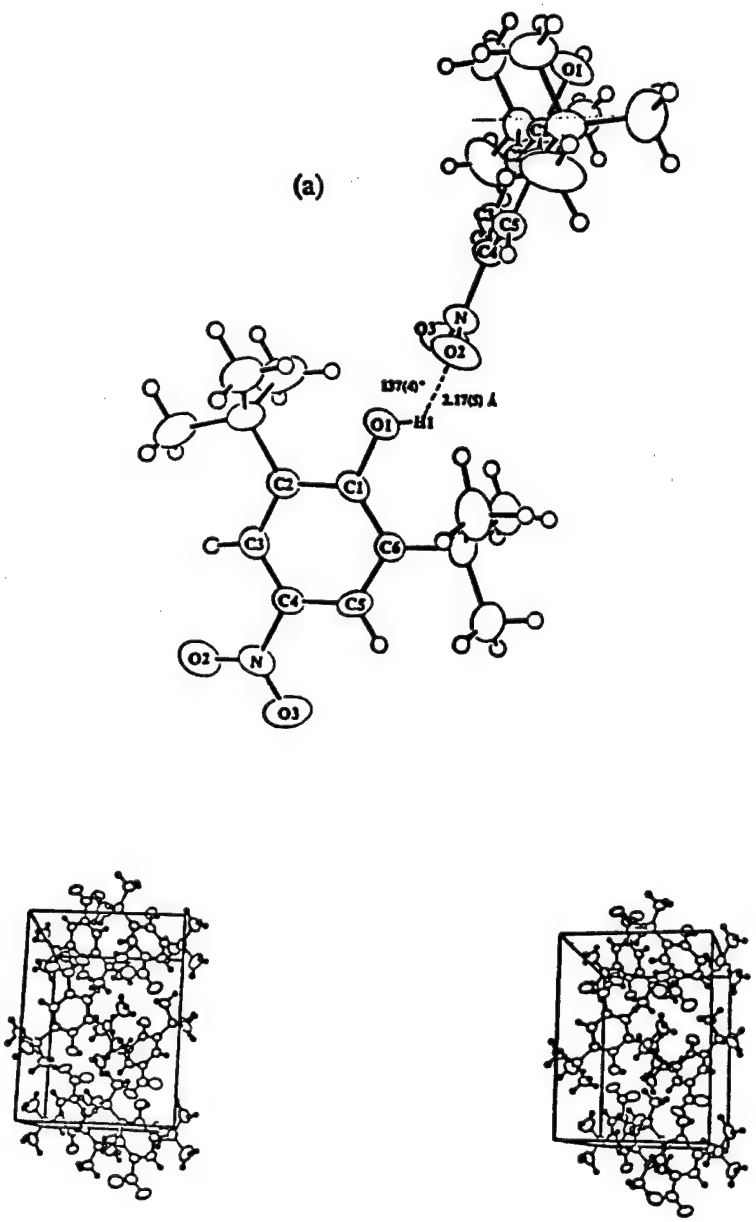


Figure 4. X-ray Crystal Structure of DBNP



soluble in organic solvents such as methanol, methylene chloride, hexane, acetone, benzene and ethanol; insoluble in water; soluble in aqueous alcohol, alkalinized water, and aqueous DMSO. DBNP has a λ_{max} of 300 nm in hexane and a λ_{max} of 320 nm in methanol and methylene chloride (Fig.5). The λ_{max} of DBNP in alkaline solution is 452 nm. The molar extinction coefficient (ε) of DBNP in organic solvents (ε_{320} = 10,092 $cm^{-1}.M^{-1}$) is threefold lower as compared to ε_{452} = 30,507 $cm^{-1}. M^{-1}$ in alkaline media. The λ_{max} at 452 is used for quantitation (Fig. 5).

Acute toxicity studies carried out by Vesselinovitch *et. al.* (1961) are summarized in Table 1. In all cases, the LD-50 is above 250 mg/kg. The oral dose is half as toxic as intraperitoneal administration in rats and guinea pigs. There is no sex difference, but rats are more sensitive to DBNP than mice or guinea pigs.

Daily administration of 10 mg/kg intraperitonealy for 60 days showed no significant effects on growth rate or on the mortality rate. During a sixty-day study, 20 mg/kg dose intraperitonealy, there was a 40% mortality, and the survivors showed decrease body weight. At a 50 mg/kg dose for sixty days, there was 100% mortality.(Vesselinovitch *et.al.* 1961)

DBNP mixed with the diet (Rockland rat diet) at 0.05% and 0.1% concentrations for 16 weeks did not show any significant effect on the growth rate. At 0.2%, DBNP half of the animals died during the first three weeks, and the survivors, both male and female, showed a significant reduction in their growth rate. DBNP in the diet caused a 25% reduction in food consumption.(Vesselinovitch *et.al.* 1961)

DBNP applied on the shaved skin of the rats (1000 mg/Kg) did not show any systemic toxicity, no mortality, nor any evidence of skin irritation.(Vesselinovitch et.al. 1961)

The effect of DBNP (10 mg/Kg, for 10 days i.p.) on the growth rate in male rats are shown in Fig. 6. There is no significant difference in growth rate between the control and treated groups.).

The ratio of urine production to water intake remains the same in both the experimental and control group (Fig.7).

The clearance of ^{14}C-DBNP from the blood, administered through i.p., i.v., and oral routes is shown in Figs. 8, 9, and 10. DBNP reached a peak blood level in 5-10 minutes when administered i.p. as compared to about an hour in the case of oral administration. The rapid

TABLE 1 Acute toxicity studies of DBNP

Species	Sex	Routes of Administration	LD_{50} (mg/Kg)
Rat	Male	ip	260
	Female	ip	270
	Male	oral	450
	Female	oral	500
Guinea Pigs	Male	ip	580
	Male	oral	800
Mice	Female	ip	850
	Male	ip	700

Vesselinovitch *et al.* 1961.

Figure 5. Spectal Characterization of DBNP

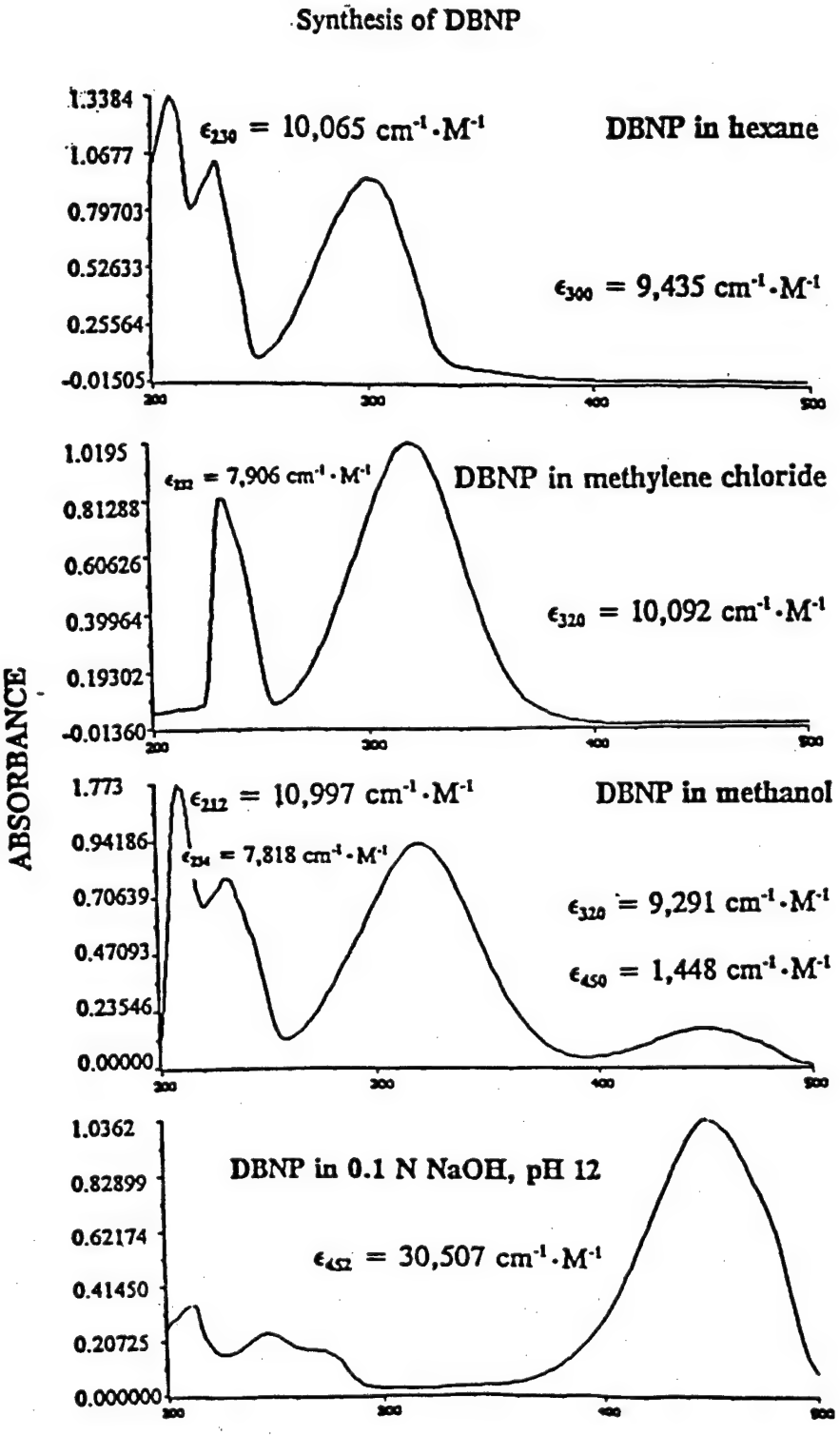


Figure 6.

Influence of DBNP on rat weight

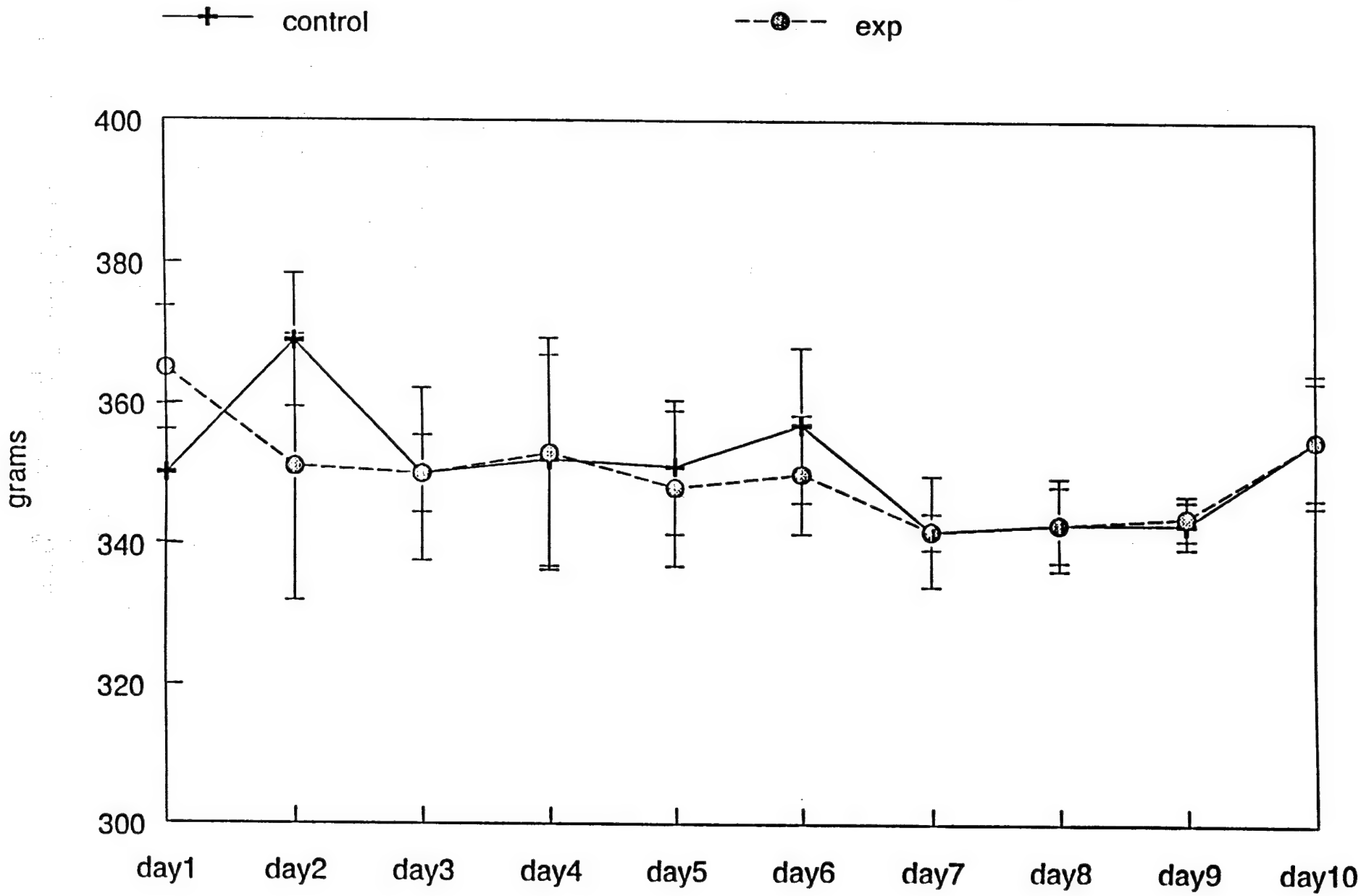


Figure 7.

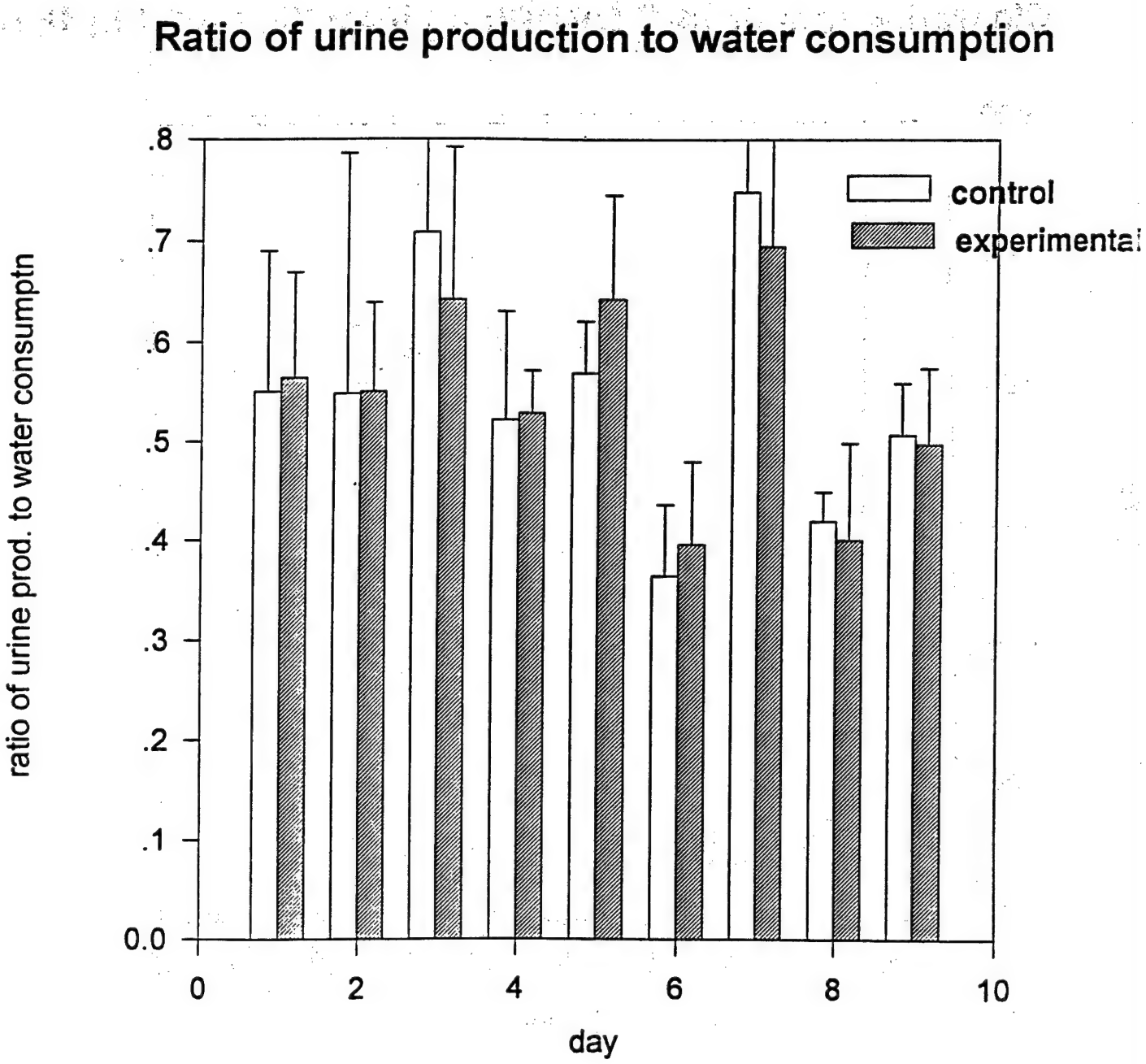

Ratio of urine production to water consumption
ratio of urine prod. to water consumptn
.8
.7
.6
.5
.4
.3
.2
.1
0.0
0
2
4
6
8
10
day
control
experimental

Figure 8.

Clearance rate of 14-C DBNP(i.p.) from the blood in 16 hour

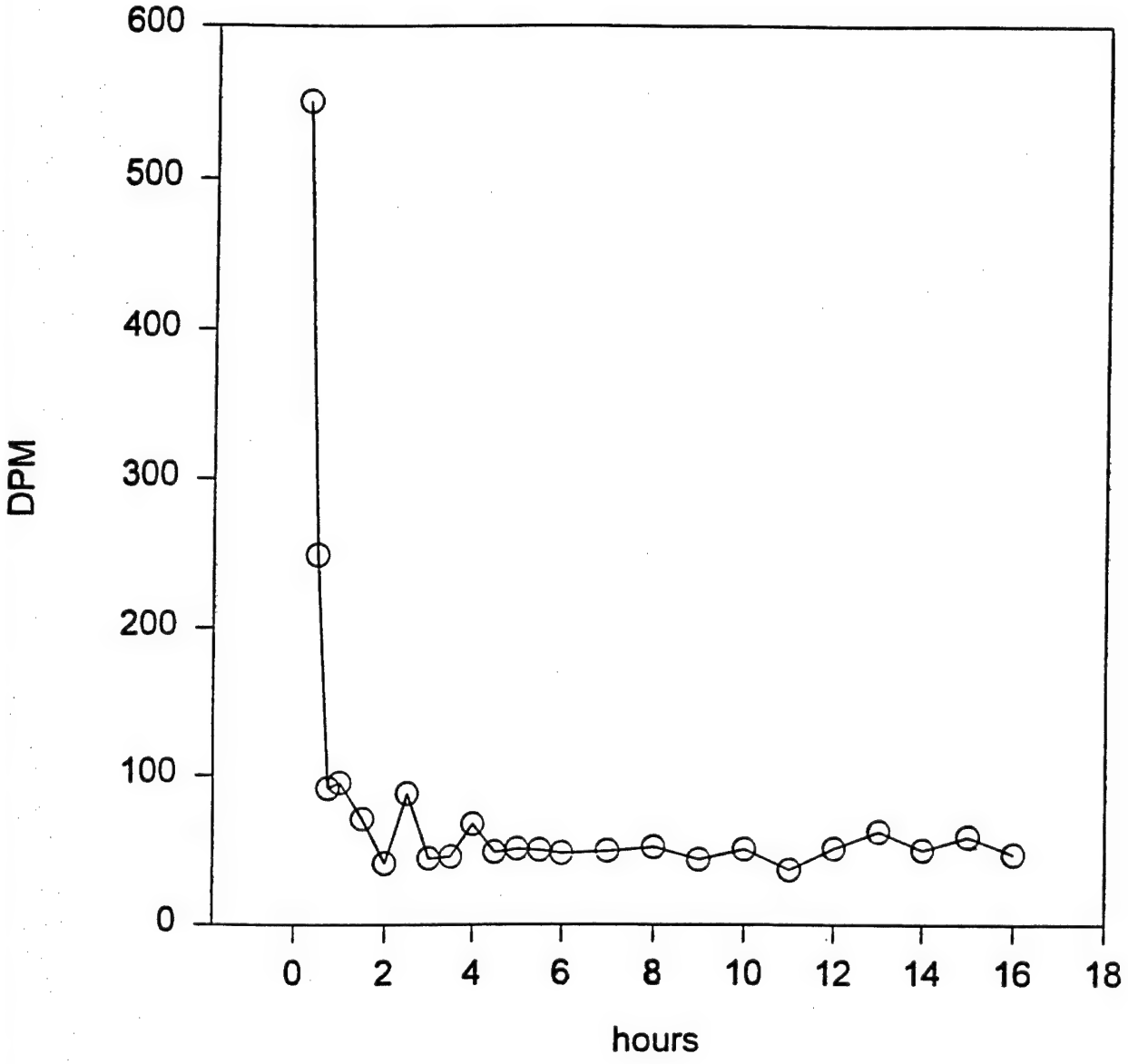


Figure 9.

Clearance of DBNP from blood, i.v.

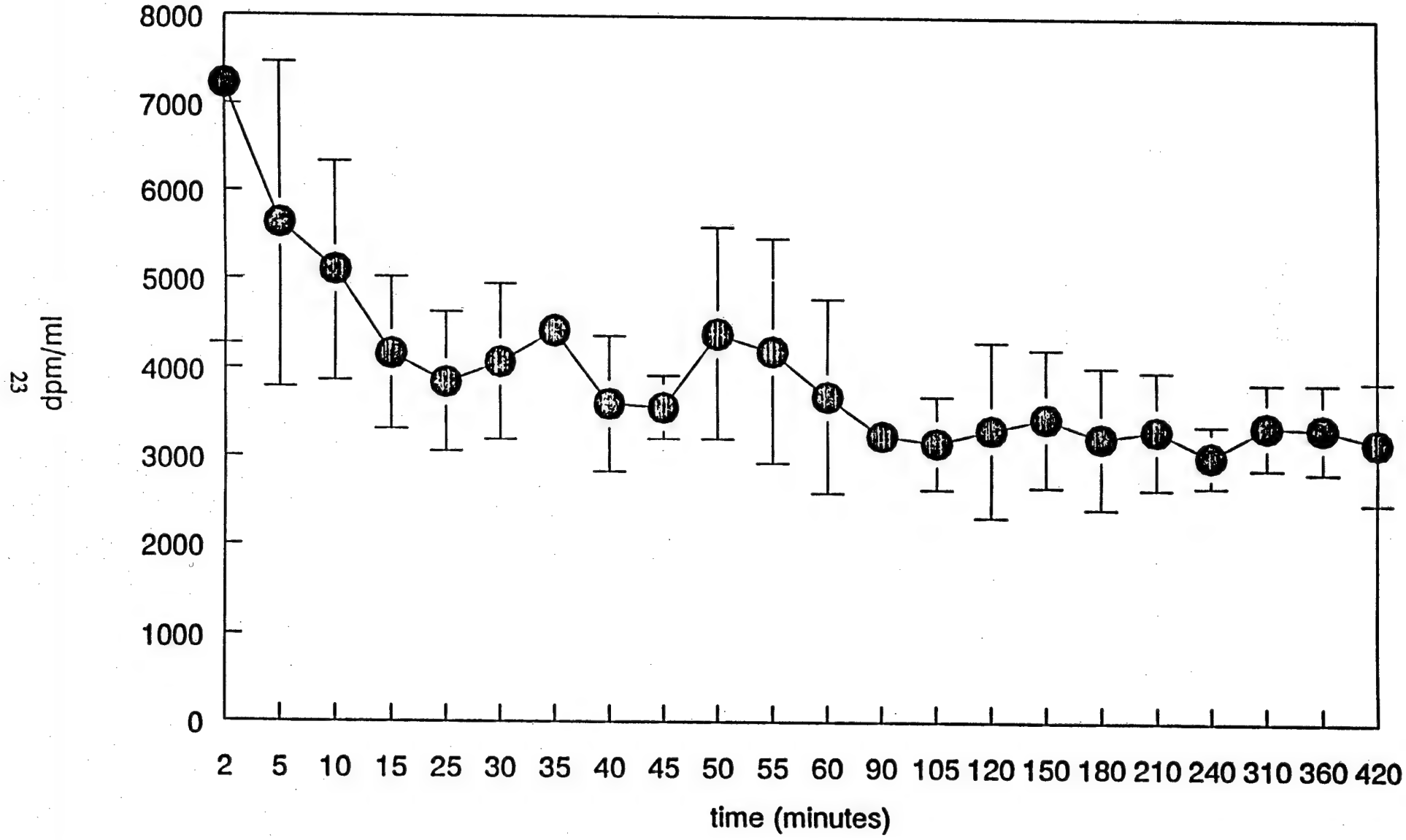
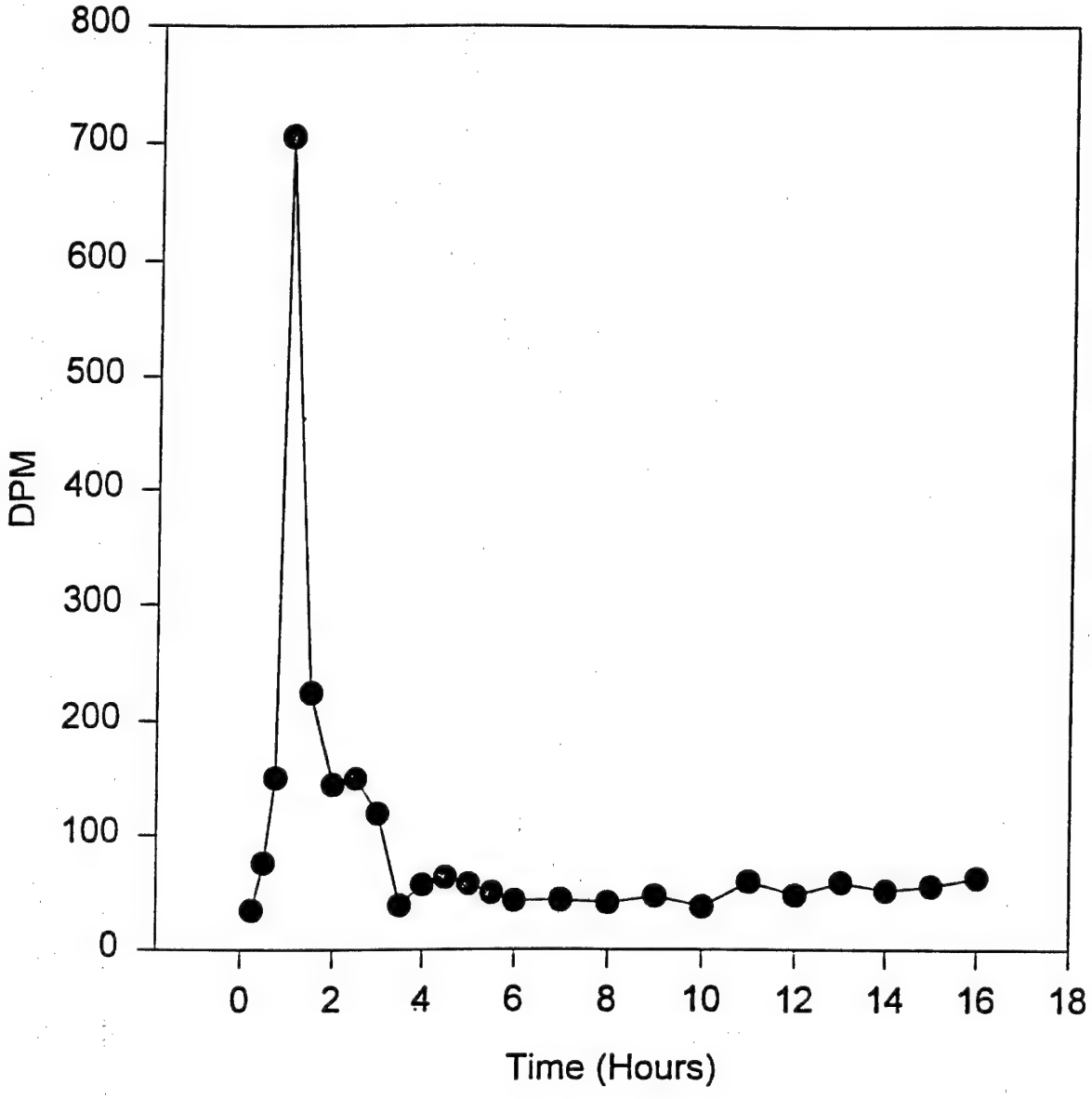


Figure 10.

Clearance of DBNP following oral dosage



phase of clearance from the blood in the first 60 minutes is followed by a steady state. This steady state continues for a week after the single initial dose and constitutes 4-6 % of the administered dose in the blood. The rapid phase of clearance is due to its distribution in all the tissues and its excretion through the urine and feces (Fig. 11a and b). Twenty four hours after a single i.p. dose of DBNP (0.4 mg/kg), 20% +/- 3.8 SD is excreted in the urine and 12% +/- 2.5 SD is excreted in the feces. The tissue distribution of DBNP 24 hours after a single dose (0.4 mg/kg) was: liver 14-16 %; spleen 3-5 %; kidney 8-10 %; heart 2-5 %; brain 0.8-1.2 %; muscle 0.5-1 %; fat 11-13 %; and blood 6-8%.

DBNP is excreted slowly from the body (Fig. 12). After a single dose of DBNP (0.4 mg/Kg), 82 -90% excreted in the urine and feces within 10 days. The excretion in the first two days averages about 18-20% for urine and 12-15% for the feces. The excretion rate in the urine and feces drop considerably from the third day (a 40% drop as compared to first two days) and continues to decrease slowly thereafter.

Urinary excretion of DBNP is lowered by 30 % in rats treated with 20 mg of Neomycin orally followed by a single oral dose of DBNP.(Vesselinovitch et.al. 1961) This suggests that intestinal microflora may contribute to the absorption of DBNP through the gastrointestinal tract after metabolic alteration by the microflora. Liver perfusion experiments clearly show that 4-5 % of the perfused amount is excreted in the bile.(Holder *et.al.* 1971)

The elution profile of the parent compound DBNP form C-18 reverse phase HPLC column detected at 450 nm is shown in Fig. 13. The retention time for DBNP is 37.55 min. under the eluting conditions described under the Materials and Methods Section.

Using identical conditions, the DBNP standard curve was generated (Fig. 14) by plotting the area under the curve against various concentration of DBNP. This standard curve is utilized to quantitate DBNP from biological samples.

The elution profile of the ^{14}C-DBNP under the identical eluting conditions is shown in Fig. 15. The difference of 2 minutes in the retention time between cold and hot DBNP is due to a delay in the starting time of the radioisotope detector after the injection plus the length of tubing between the column to the flow cell in the radioisotope detector.

Figure 11A.

Amount of radioactive label present in tissues 24 hrs post-exposure to DBNP

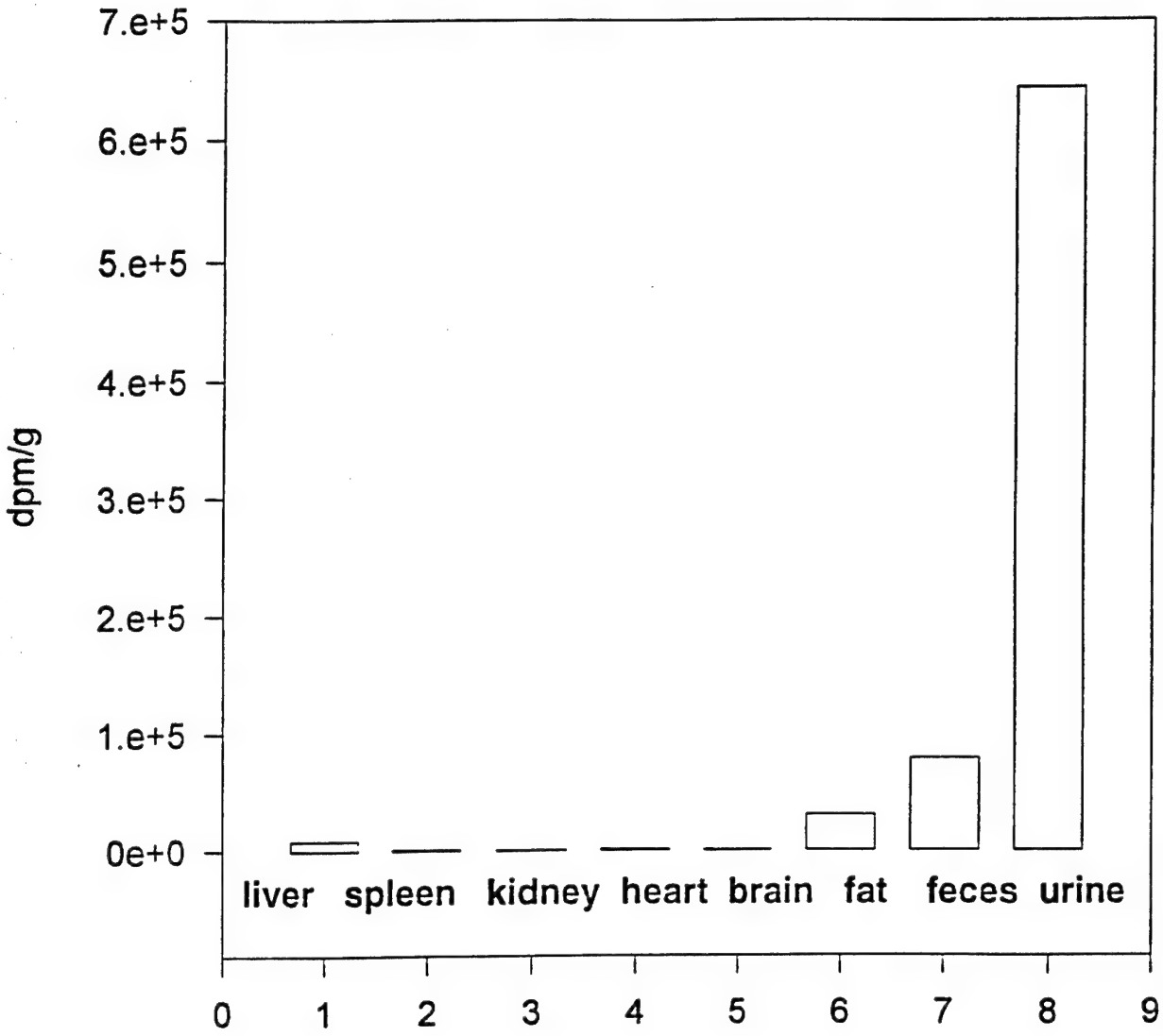


Figure 11B.

Distribution of DBNP in the tissues after i.p. injection

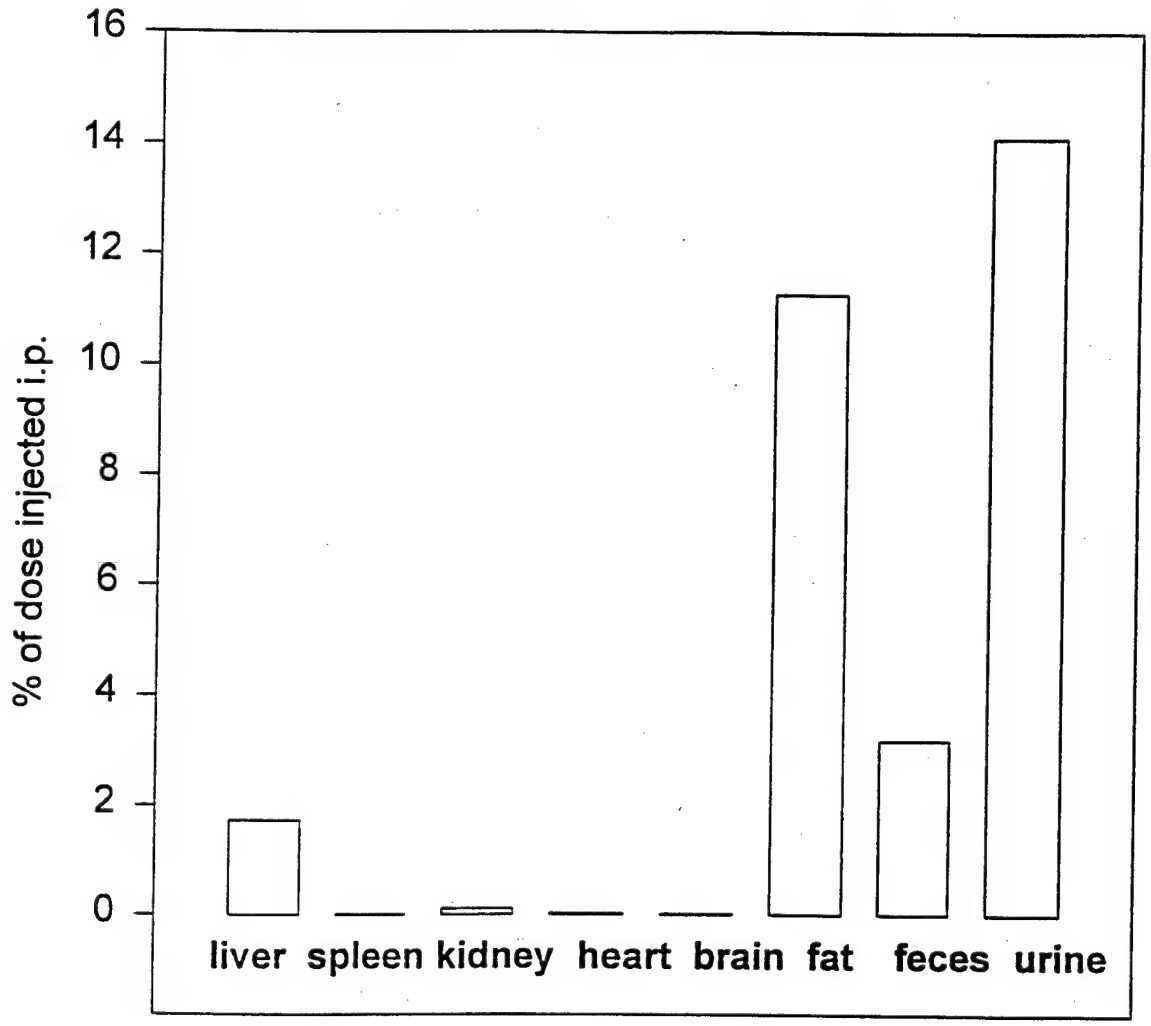


Figure 12.

Elimination of ^{14}C-DBNP in the urine and feces

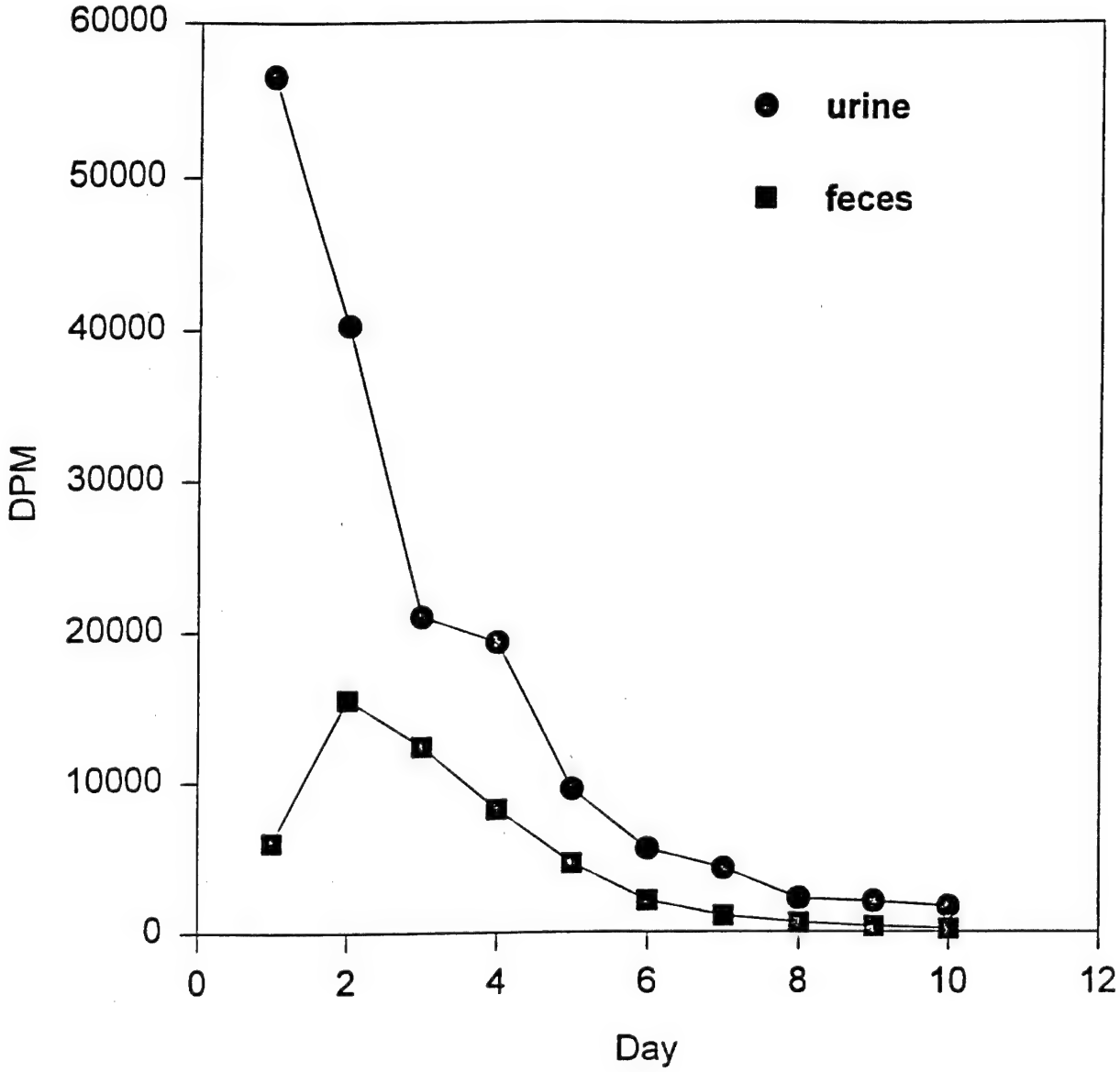


Figure 13
Elution Pattern of DBNP from HPLC Chromatography

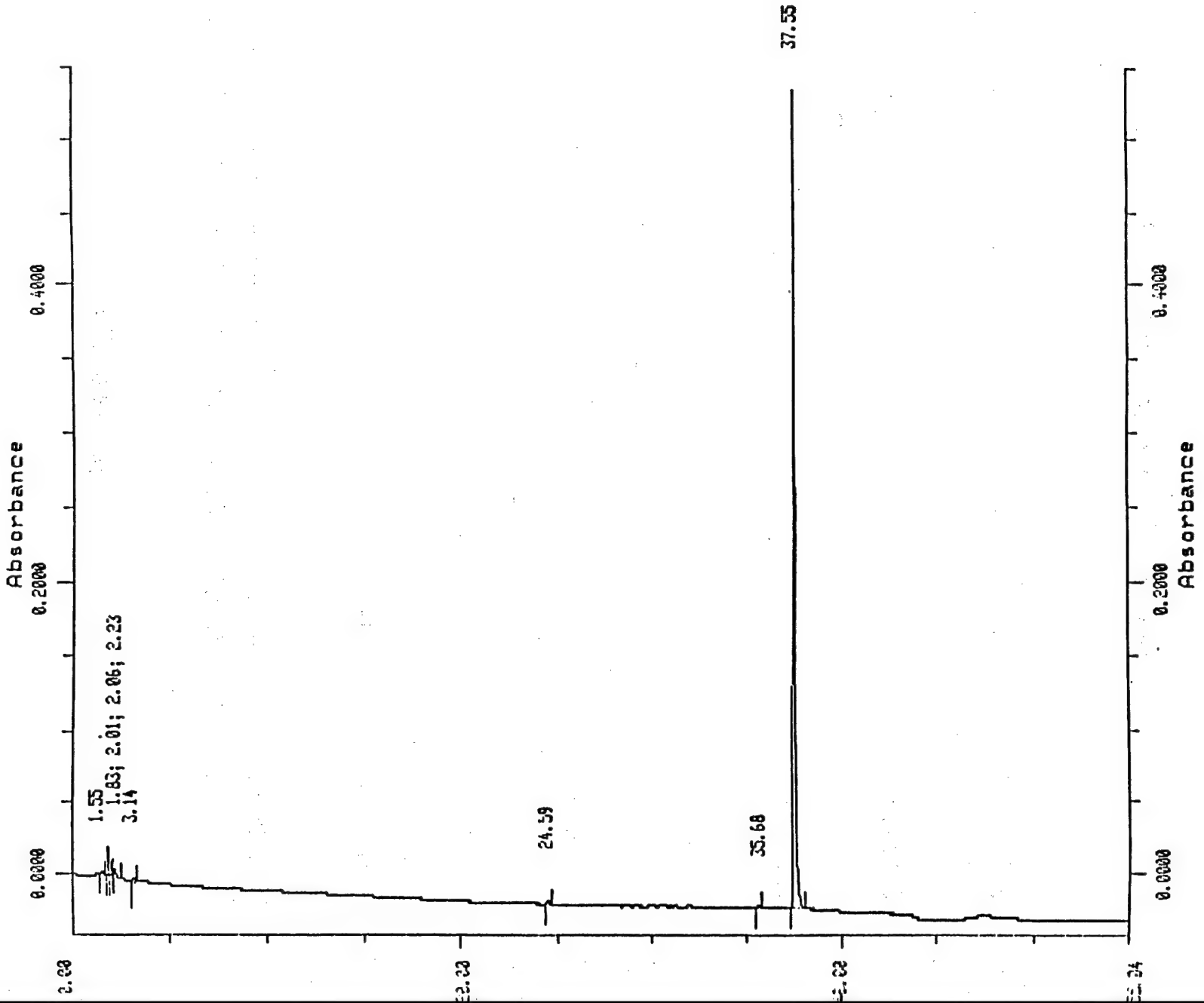


Figure 14.

Standard curve of the area of the DBNP peak via HPLC.

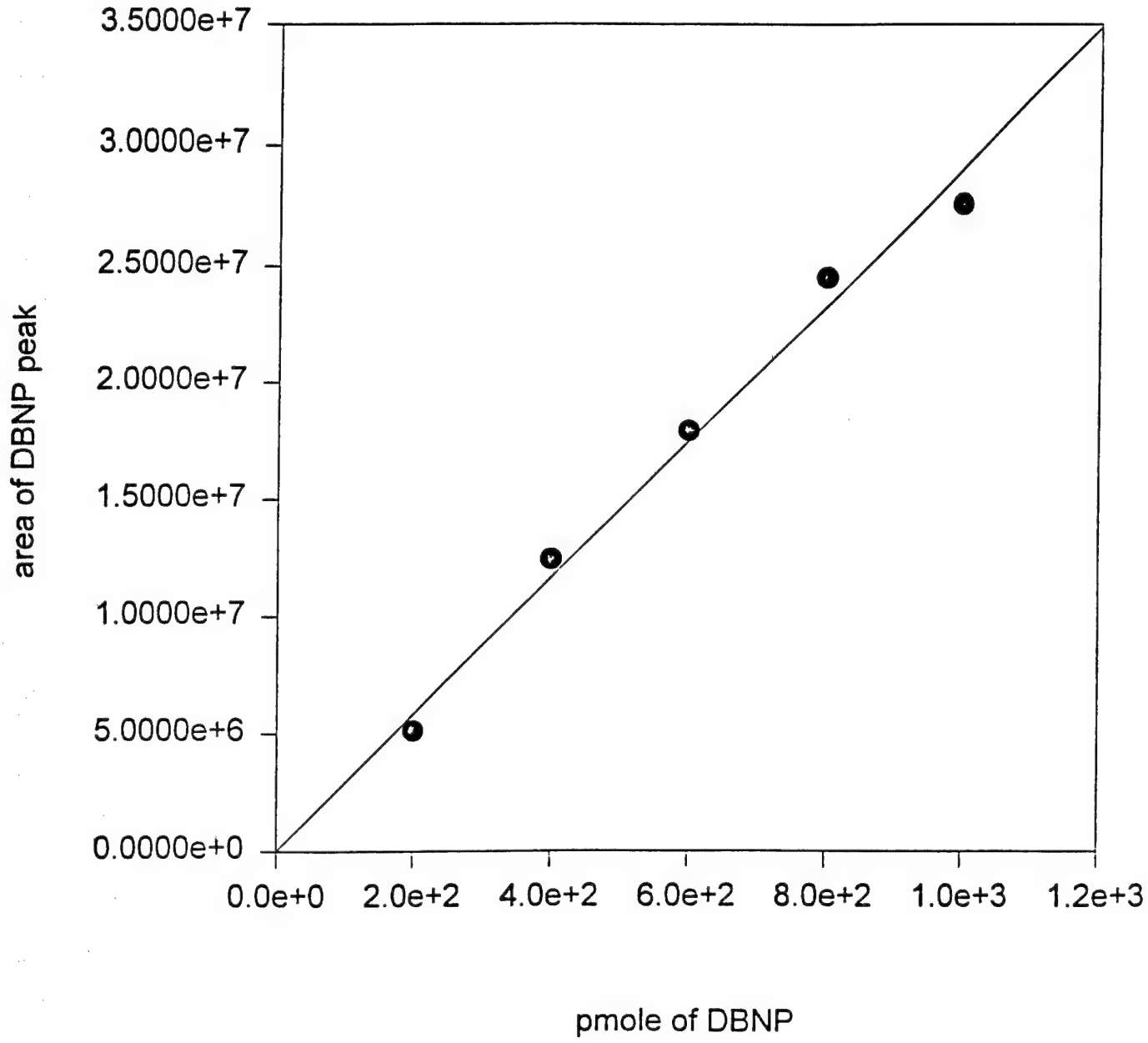
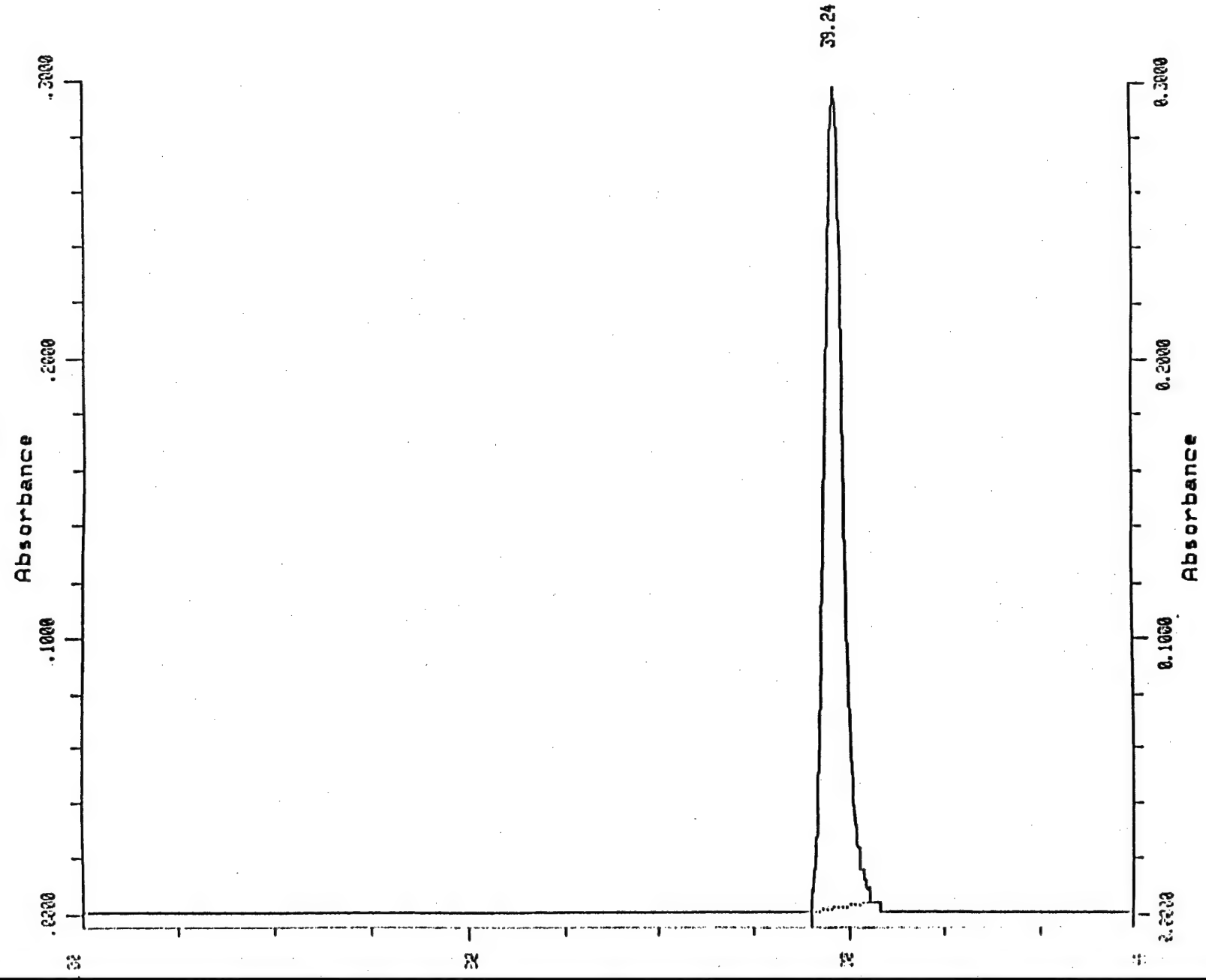


Figure 15
Elution Pattern of ^{14}C-DBNP From HPLC Chromatography



The elution profile of the metabolite isolated from the urine and feces is shown in Figs. 16 and 17. The metabolite was eluted earlier (25 minutes) than the parent compound DBNP (37.55). No parent compound DBNP are any other metabolite was detected in the purified samples from the urine and feces. The metabolite isolated from bile also eluted as a single peak in HPLC with the same retention time as the urine and feces sample.

The purified material from the urine, feces and bile was identified as a glucuronide conjugate after the radioactive material was digested with glucuronidase. The glucuronide conjugate DBNP is of the ether type. Generally the ether glucuronides are unstable in acidic conditions. After acid hydrolysis under nitrogen, the liberated glucuronic acid was isolated and crystallized. The presence of glucuronic acid was further confirmed by its melting point, reduction of alkaline cooper sulfate and naphthoresorcinol color reaction. Failure to identify unchanged DBNP in the urine and in the bile indicated that, once DBNP is aborbed, it is excreted after phase-II metabolism to a glucuronide conjugate. Acid hydrolysis of the metabolite followed by HPLC failed to show the presence of any other metabolite in which the nitro group is reduced.

Toxicity of DBNP at varied concentrations on rat and human liver slices is shown in Figs. 18 and 19. Comparison of nitrophenol toxicity in rat and human liver slices is shown in Table 2. Rat liver slices are more sensitive to DBNP insult than human liver slices. The order of susceptibility of both species was ATP content > protein synthesis > LDH release > K^+ leakage.

At a 50μM concentration, DBNP produced a 70% reduction in ATP content in rat liver slices as compared to a 30% reduction in human liver slices. At the same concentration, protein synthesis was reduced by 60% in rat liver slices as compared to 30% in human liver slices. At higher concentrations (200 μM), the toxic effects of DBNP are seen within an hour; whereas, at lower concentrations (25 μM), the toxic effect is seen after 4 hours.

Hepatocyte cells grown in tissue culture medium lose thier viability (100%) when exposed to DBNP at 2μg / ml concentration for 24 hours. Cells exposed to DBNP at 0.1 and 0.2μg /ml concentrations for 24 hours show a viability loss of 8% and 15%, respectively.

The effect of DBNP on state-3 and state-4 mitochondrial respiration are shown in Figs. 20 and

Figure 16. DBNP metabolite from urine

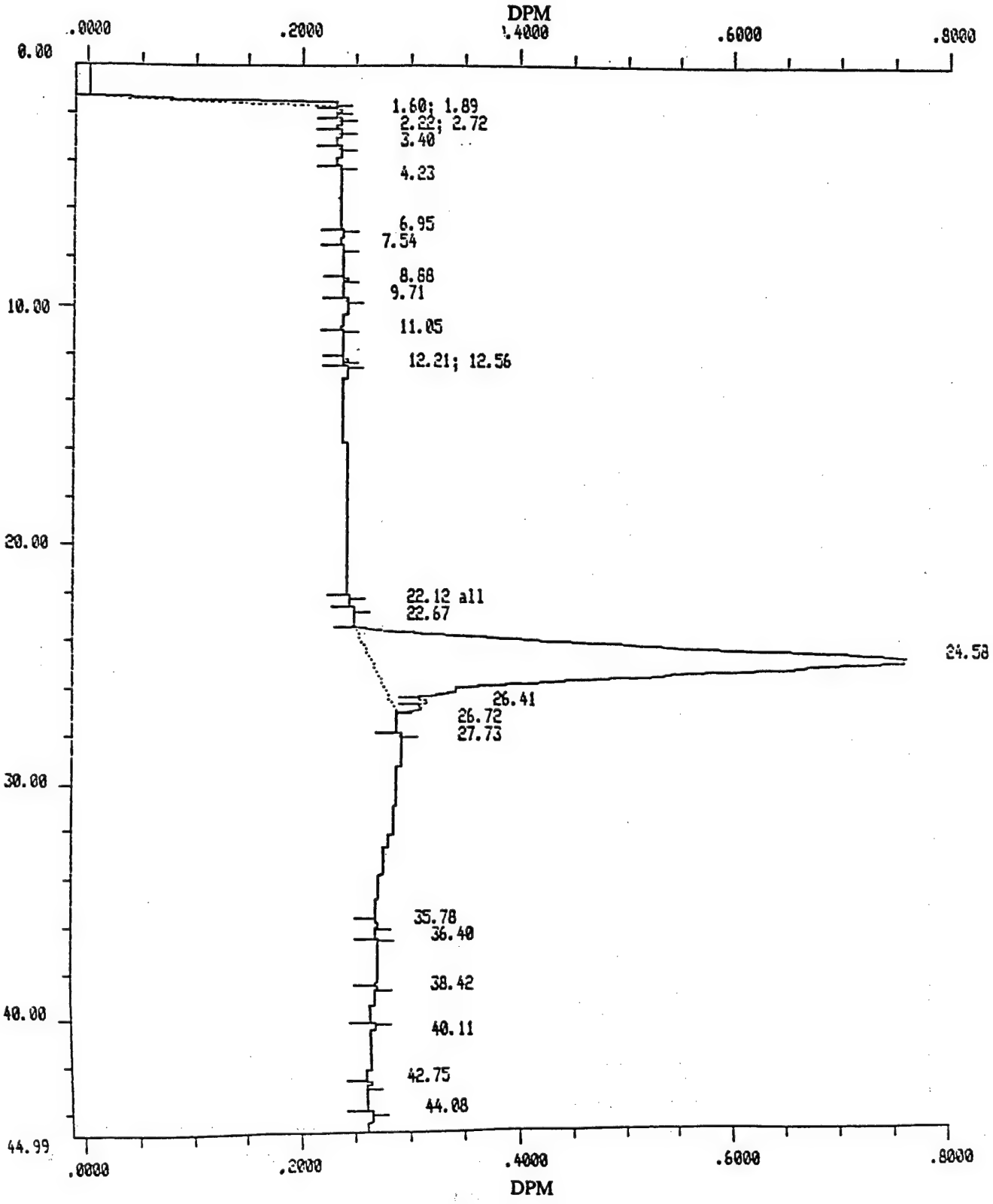


Figure 17. DBNP metabolite from feces

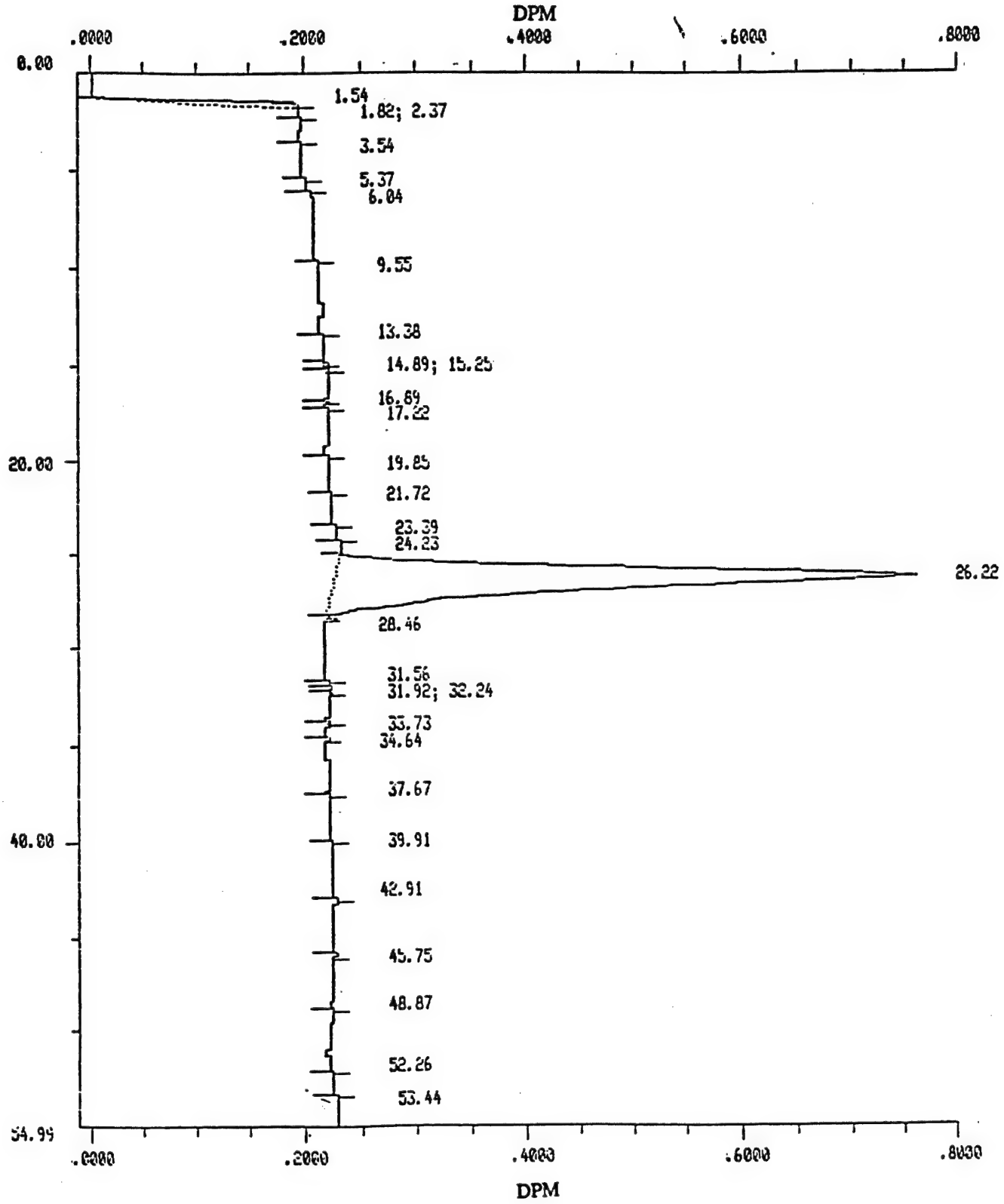


Figure 18. Toxicity of Varied concentrations of DBNP to Rat Liver Slices after Two Hours Incubation

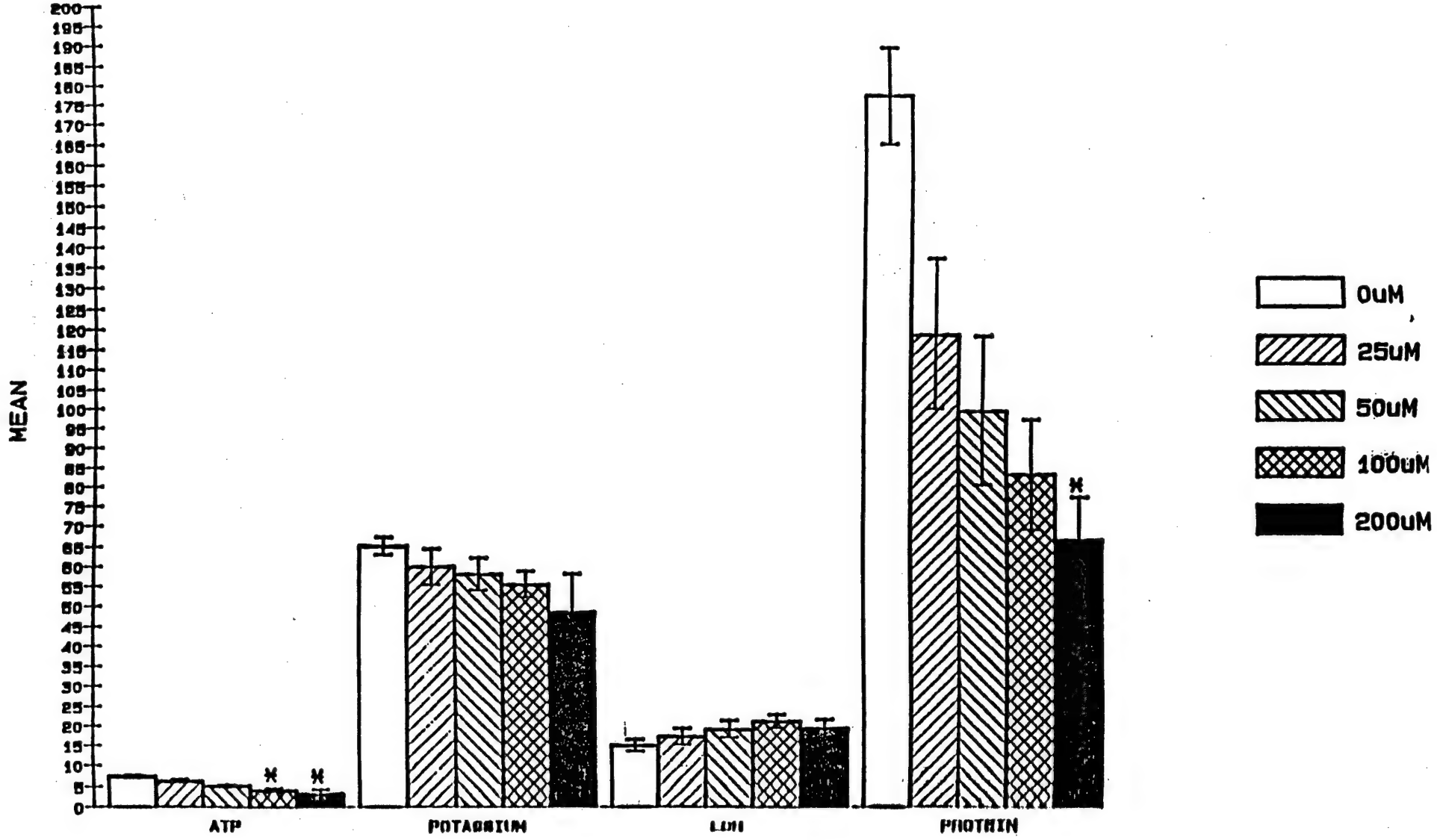


Figure 19. Toxicity of Varied Concentrations of DBNP to Human Liver Slices after Two Hours Incubation

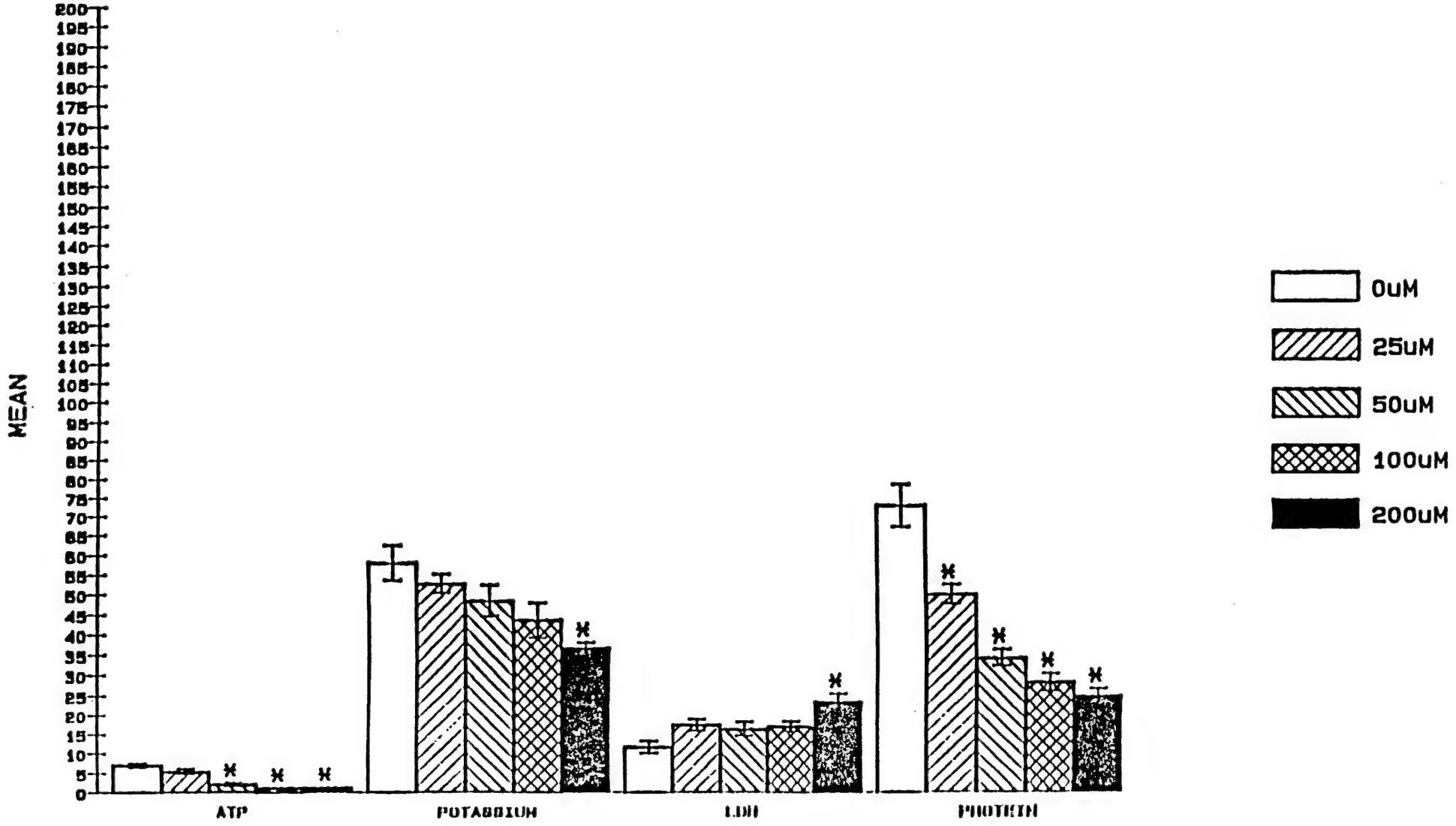


TABLE 2. Comparison of Nitrophenol Toxicity in Rat and Human Liver Slices.

Nitrophenol (50 uM)	RAT ATP Content[a]	RAT Protein Synthesis[b]	HUMAN ATP Content[a]	HUMAN Protein Synthesis[b]
Control	5.9 ± 0.7[c]	112.7 ± 23.2[c]	7.2 ± 0.4	177.3 ± 21.1
DN-*t*-BP	2.2 ± 0.0*	57.0 ± 19.3*	3.8 ± 1.7*	77.0 ± 12.2
Control	7.0 ± 0.7	73.0 ± 9.6	———	———
DBNP	2.2 ± 0.7*	34.3 ± 3.5*	5.1 ± 0.4	99.0 ± 32.7
2,4-DNP	6.1 ± 0.2	57.3 ± 28.0	6.5 ± 0.8	156.3 ± 26.8
4-NP	7.0 ± 0.6	79.0 ± 16.5	7.3 ± 1.2	142.7 ± 19.4

Values represent the mean ± S.D. of three determinations. The concentration and exposure time selected for comparison were 50 uM and 2 Hr, respectively.

a) nmo/mg wet wt.

b) DPM/mg wet wt.

c) Control values for DN-*t*-BP exposures were different from the control used for the other nitrophenols evaluated because these studies were conducted at a different time.

* Statistically different from control values; $p \geq 0.05$.

21. Both DBNP and DNP (a potent uncoupler of oxidative phosphorylation) inhibited mitochondrial respiration and ATP production. DBNP is one-third less potent than DNP inhibiting ATP synthesis in mitochondrial preparations.

DBNP (200 μ M) has no effect on FABP and sulfotrasferases in vitro.(Appendix-3, results, page-9). A slight increase in FABP was seen in DBNP perfused liver as compared to normal. (Table-1/ Appendix-c). No change in BST level was seen in DBNP perfused rat liver. (Table-2/ Appendix-c). In sub-accute toxicity studies the survived rats after 30 days (25mg/kg daily/i.p) showed decrease in body weight, increase in liver weight and increase in liver weight/body weight ratio.(table-3 / Appendix-c). Signifant changes were seen in the level OF FABP, DBT and BST in DBNP treated rats.

Figure 20.

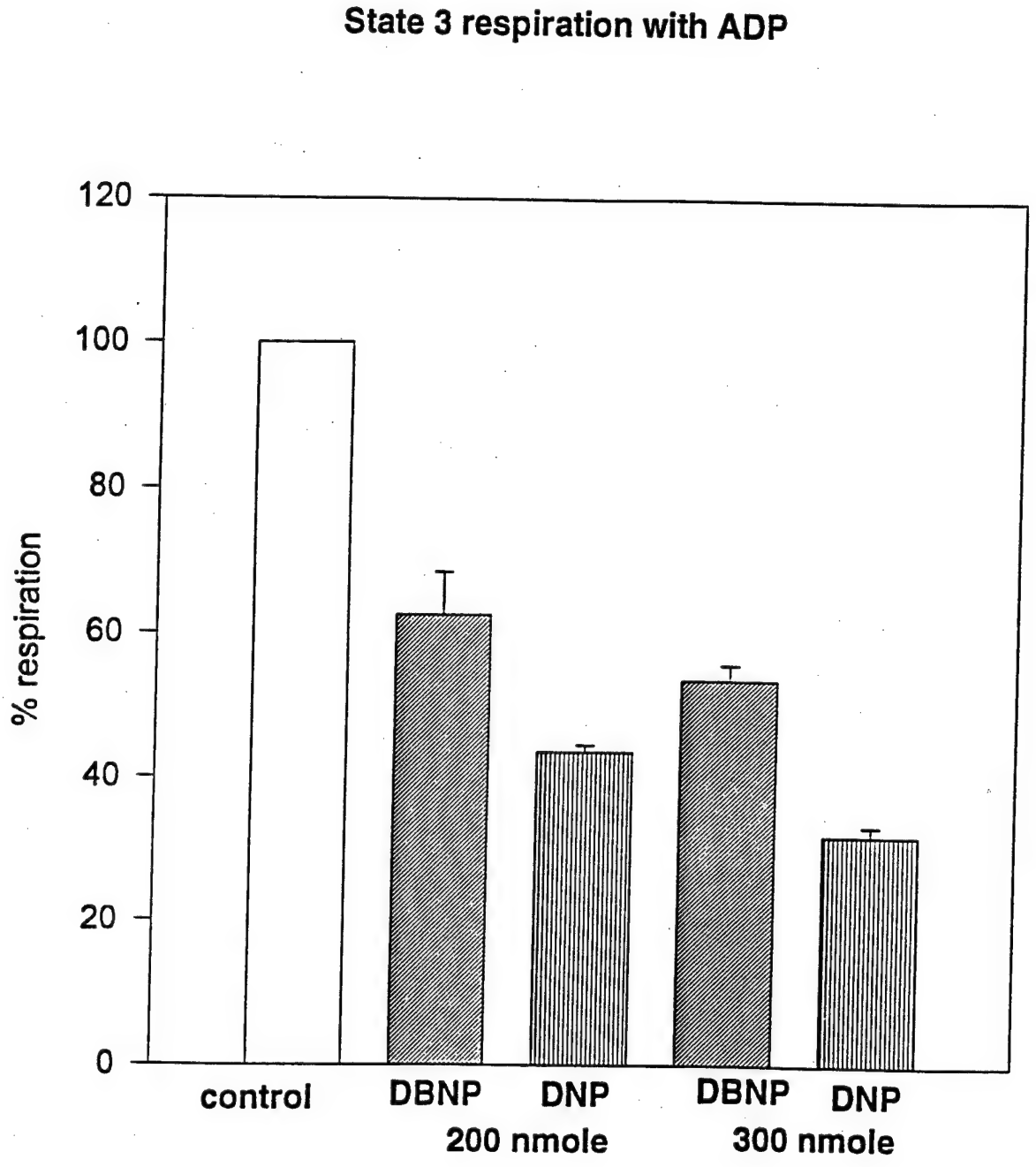
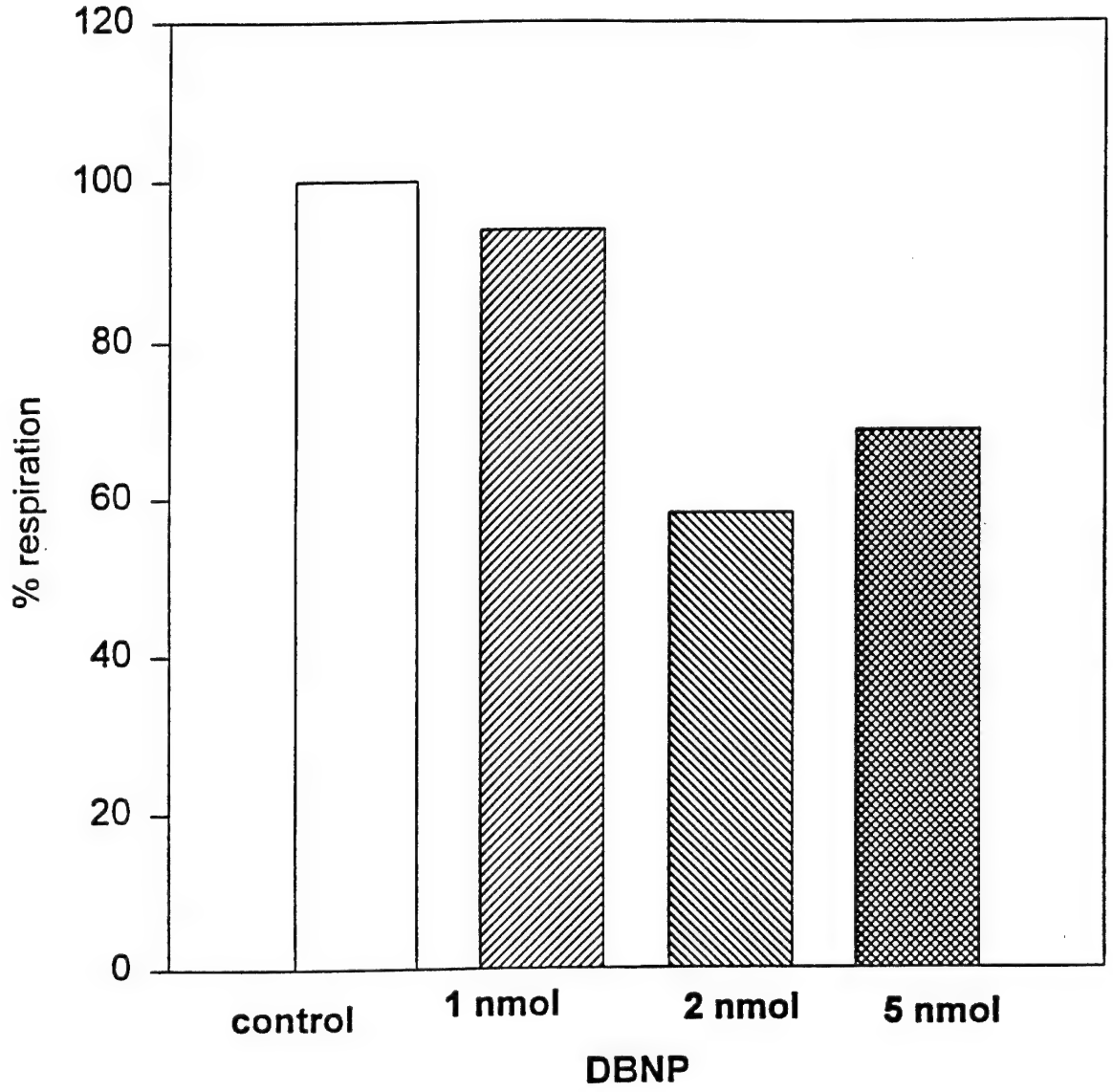

State 3 respiration with ADP
% respiration
120
100
80
60
40
20
0
control
DBNP
DNP
DBNP
DNP
200 nmole
300 nmole

Figure 21.

State 4 respiration with Succinate



SECTION 4

CONCLUSIONS

Combining literature information with the studies conducted under this work unit, one can draw the following conclusions concerning DBNP toxicity. The LD-50 dose by intraperitoneal injection in rodents is above 250 mg/kg, indicating that DBNP is of low toxicity. The oral dose was half as toxic as intraperitoneal administration in rats and guinea pigs. There is no sex difference, but rats are more sensitive to DBNP than other animals. High doses of DBNP applied on the rat skin did not show any toxicity or evidence of skin irritation, which suggests that there is low risk in dermal exposure.

Even though these acute toxicity test demonstrated that DBNP has a low toxicity in rats, mice, and guinea pigs, DBNP has a considerable tendency to produce cumulative toxic effects when administered repeatedly at one-tenth of the LD-50 dose. These observations suggest a potential health risk in situations where personnel may be repeatedly be exposed to even low doses of DBNP. DBNP is cleared from the system very slowly, and repeated exposures may slow the excretion further. These factors, combined with the fact that a considerable amount of DBNP is present in the fat and that the excretion rate drops after the first few days, contribute to the observed cumulative toxicity. The drop in the excretion rate may also be due to slow mobilization from the fat giving rise to a reduced level in the general circulation. The drop in the excretion rate is not due to reduced kidney function because water consumption and urine output remained the same as control. It is very likely that enterohepatic circulation of DBNP and its metabolite (see below) contributes to the delayed elimination of DBNP from the system.

DBNP is excreted in the bile as a glucuronide conjugate but not as a sulfate conjugate. HPLC analysis showed the presence of a single metabolite from the urine, feces or bile. Acid hydrolysis failed to show the presence of any other metabolite in which the nitro group is removed or reduced.

At the cellular level, the toxicity expressed by DBNP is very likely due to its inhibitory effect on

ATP synthesis, a well-known action of nitrophenols in general that leads to the inhibition of the number of biochemical pathways (anabolic and catabolic), particularly energy- dependent enzymes in carbohydrate metabolism. These biochemical actions lead to the fatty patches seen in the liver at higher does. In-vitro experiments with human liver slices indicate that humans may be less sensitive to the toxic effects of DBNP than other species (within the parameters studied thus far). With both in vitro and perfused liver experiments, DBNP has no effect on sulfotransferases and FABP, although repeated i.p. injections of DBNP (25 mg/kg; 30 days) caused an increase in FABP and a decrease in BST in-vivo. Thus, while in-vitro exposure to DBNP affects these fatty acid transport and metabolic factor, one cannot rule out the possibility that the histopathalogical changes seen in the tissues at high doses of DBNP may be due to its effect on other biochemical processes in combination with the inhibition of ATP synthesis.

SECTION 5

PUBLICATIONS AND PRESENTATIONS

1. J.A.Rivera, J.F.Wyman, D.L.Von Minden, N.Lacy, M.L. Chabinic, A.V. Fratini and D.A. Macys. (1995) Facile synthesis and physical and spectral characterization of 2,6-di-t-butylnitrophenol (DBNP): A potentially powerful uncoupler of oxidative phosphorylation. *Environ. Toxicol.Chem.* 14: 251-256.

2 J.Wyman, N.Reo, J.Rivera, T.Moore, S. Prues, D.Lee, C. Goecke and C. Alva. (1994) Hepatotoxicity. of 2,6-di-t-butyl-4-nitrophenol to isolated-perfused rat liver. *The Toxicologist* 14: 681.

3. J.F. Wyman, R.Fisher, S.L.Prues, C.D. Fleming and K. Brendel (1995) Comparative toxicity of 2,6-di-t-butyl-4-nitrophenol and other nitrated phenols in human and rat hepatic tissue slices. *The Toxicologist* 15: 1532.

4. S.S. Singer, M.Cunningham, T.Jewett and J.Wyman.(1995) Subacute 2,6-di-t-butyl-4-nitrophenol (DBNP) effects on the rat liver fatty acid binding protein and bile salt/dopamine sulfotranferase. *The Toxicologist* 15: 1689.

5. Synthesis and spectral properties of 2,6-di-*t*-butyl nitrophenol (March 15, 1994). Presented at the *American Chemical Society* 207th meeting held at Navy Post-Graduate Medical School, Monterey, CA.

6. Comparative toxicity of 2,6-di-*t*-butyl-nitrophenol and other related phenols in human and rat hepatic tissue slices. (March 2, 1994) Presented at the *Thirty fifth Navy Occupational Health and Preventive Medicine (NOHMP) workshop*. Held at Navy Environmental Health Center, Norfolk VA.

7. Subacute 2,6-di-t-butyl-4-nitrophenol (DBNP) effects on the rat liver fatty acid binding protein and bile salt/dopamine sulfotransferases. (March 12, 1995) Presented at *Society of Toxicolody Annual Meeting* held at Baltimore, MD.

MANUSCRIPT SUBMITTED

1. Absorption, Distribution, Metabolism, and Excretion of 2,6-Di-t-butyl-4-nitrophenol in Fischer-344 Rats. T.K. Narayanan, A.E. Jung, S.L. Prues, R.L. Carpenter, and K.R. Still. Submitted to *Toxicology Letters*.

2. Toxicity of 2,6-di-*t*-butyl-nitrophenol in human and rat liver slices preparation. J.F. Wyman *et. al. In Vitro Toxicology*.

SECTION 6

REFERENCES

1. Appendix A (attached)

2. Appendix B (attached)

3. D. Vesselinovitch, Kenneth P. DuBois, F.W. Fitch and John Doull **(1961)** Mammalian toxicity and histopathalogical effects of 2,6-dibutyl-4-nitrophenol. *Toxicology and Applied Pharmacology.* **3 713-725**

4. G.M.Holder, A.J. Ryan, T.R. Watson and L.I. Wiebe **(1971)** *Food and Cosmetic Toxicology.* **9, 531-535**

5. **1**.J.A.Rivera, J.F.Wyman, D.L.Von Minden, N.Lacy, M.L. Chabinic, A.V. Fratini and D.A. Macys.**(1995)** Facile synthesis and physical and spectral characterization of 2,6-di-t-butylnitrophenol (DBNP): A potentially powerful uncoupler of oxidative phosphorylation. *Environ. Toxicol.Chem.* **14:** 251-256

6. J.Wyman, N.Reo, J.Rivera, T.Moore, S. Prues, D.Lee, C. Goecke and C. Alva. **(1994)** Hepatotoxicity of 2,6-di-t-butyl-4-nitrophenol to isolated-perfused rat liver. *The Toxicologist* **14:** 681

7. J.F. Wyman, R.Fisher, S.L.Prues, C.D. Fleming and K. Brendel **(1995)** Comparative toxicity of 2,6-di-t-butyl-4-nitrophenol and other nitrated phenols in human and rat hepatic tissue slices. *The Toxicologist* **15:** 1532

8. S.S. Singer, M.Cunningham, T.Jewett and J.Wyman.**(1995)** Subacute 2,6-di-t-butyl-4-nitrophenol (DBNP) effects on the rat liver fatty acid binding protein and bile salt/dopamine sulfotranferase. *The toxicologist* **15:** 1689

Appendix A

NRL EFFORTS ON THE

SUBMARINE SURFACE YELLOWING PHENOMENON

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NRL Involvement

Introduced to the problem and asked to get involved (early May)

Draw upon extensive submarine atmosphere (and materials) a lysis experience, as well as analytical resources

Learned of the knowledge to date, especially the important, initi work of EB

Mobil oil contains 2,6-di-t-butyl-phenol (DBP) and 2,6-di-t-but 4-methylphenol (DBMP or BHT)

2,6-di-t-butyl-4-nitrophenol (DBNP) identified (CAS #728-40)

Questions

1. What is causing the surface yellowing onboard some submar es?
2. Is the nitrophenol (DBNP) identified by EB responsible?
3. How is the DBNP formed, in what quantities and where?
4. What are the precursors and what levels are found airborne nd on surfaces?
5. What are some possible solutions to this problem?

Sampling

Rode USS Maryland (SSBN 738) (May 26/27)
    Acquired air (closed boat), surface swipe and oil samples
Received surface scraping samples from SSN 724
Visited EB and USS Annapolis (SSN 760) (June 11/12)
    Acquired air (open boat) and surface swipe samples
Plan to ride USS Annapolis (June 30)

Laboratory work

Analysis of submarine samples

Thermal desorption of Tenax air samples
Solvent (CH_2Cl_2) extraction of surface swipe samples, scrapings or oils
Gas chromatography/mass spectrometry (GC/MS) analysis

Other experiments:

Exposure of phenols to NO_2
Attempts to sample airborne nitrophenol
Quantitation of airborne phenols

Results of Submarine Air Sample Analyses

USS Maryland (SSBN 738) (closed boat samples)

All samples showed DBP and DBMP, with increasing concentrations in more aft locations; much more DBP aft
The concentrations of the phenols were estimated to be XXX ppm
No samples yielded any detectable amount of DBNP

USS Annapolis (SSN 760) (open boat samples)

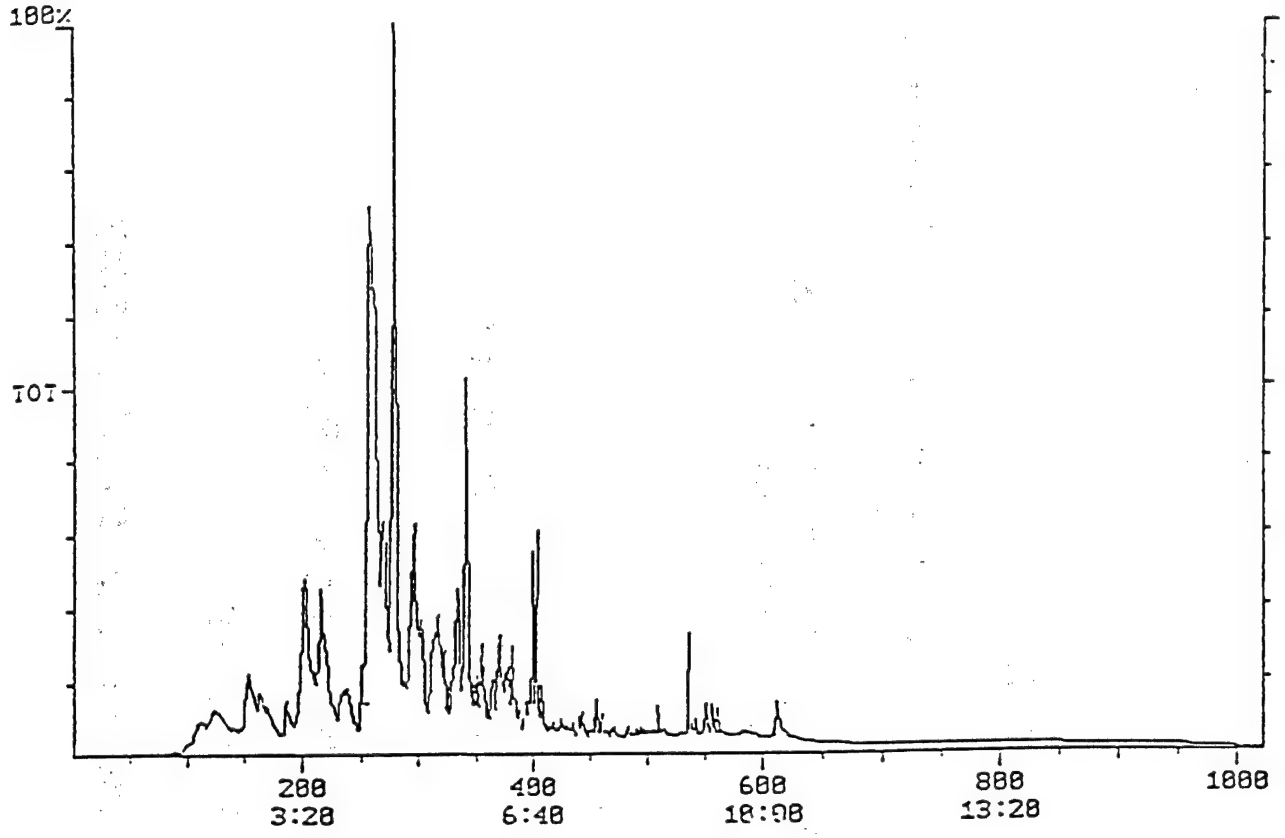
As on SSBN 738, DBMP but no DBP or DBNP were detected

Previous Submarine Air Sampling Trips - Not Targeting Phenols

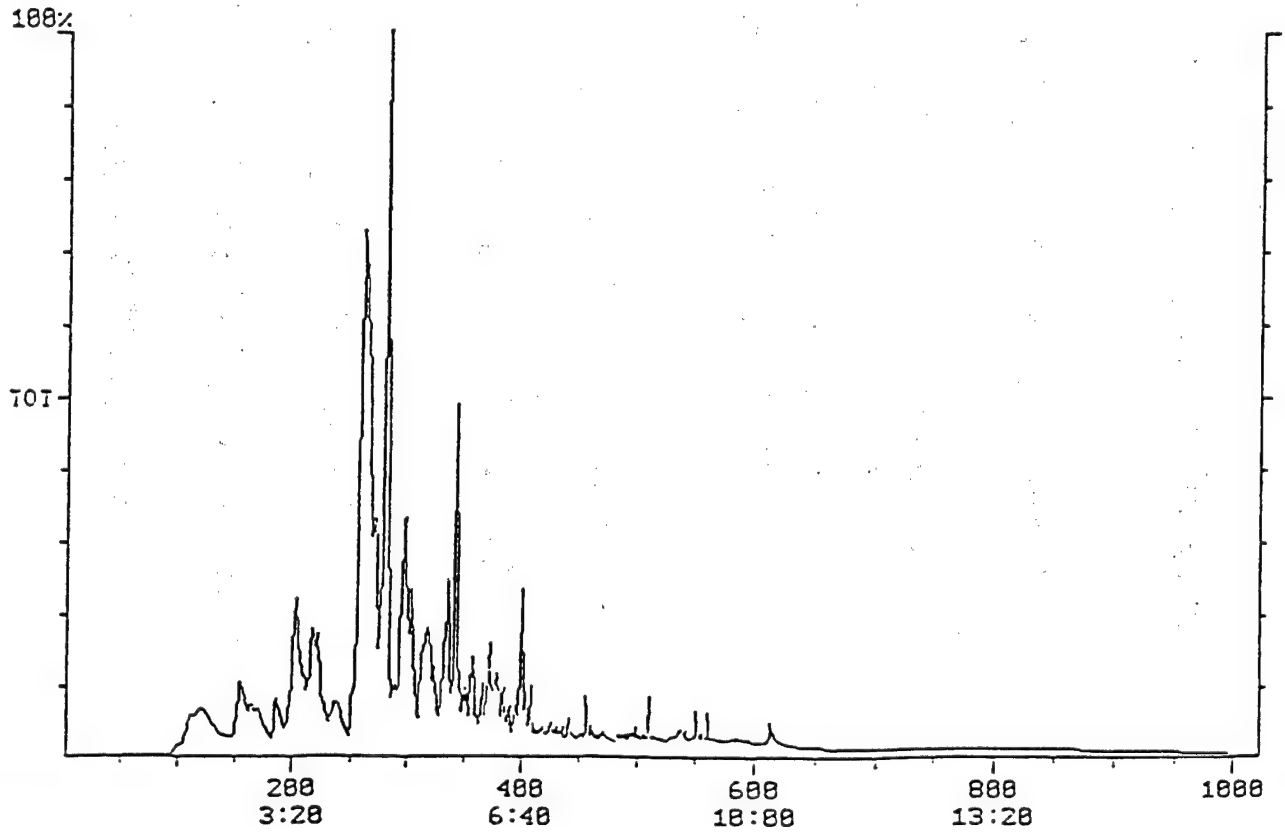
USS Pargo (SSN 650), USS Alabama (SSBN 731)

Airborne DBMP detected (background?) but no DBNP or DBP

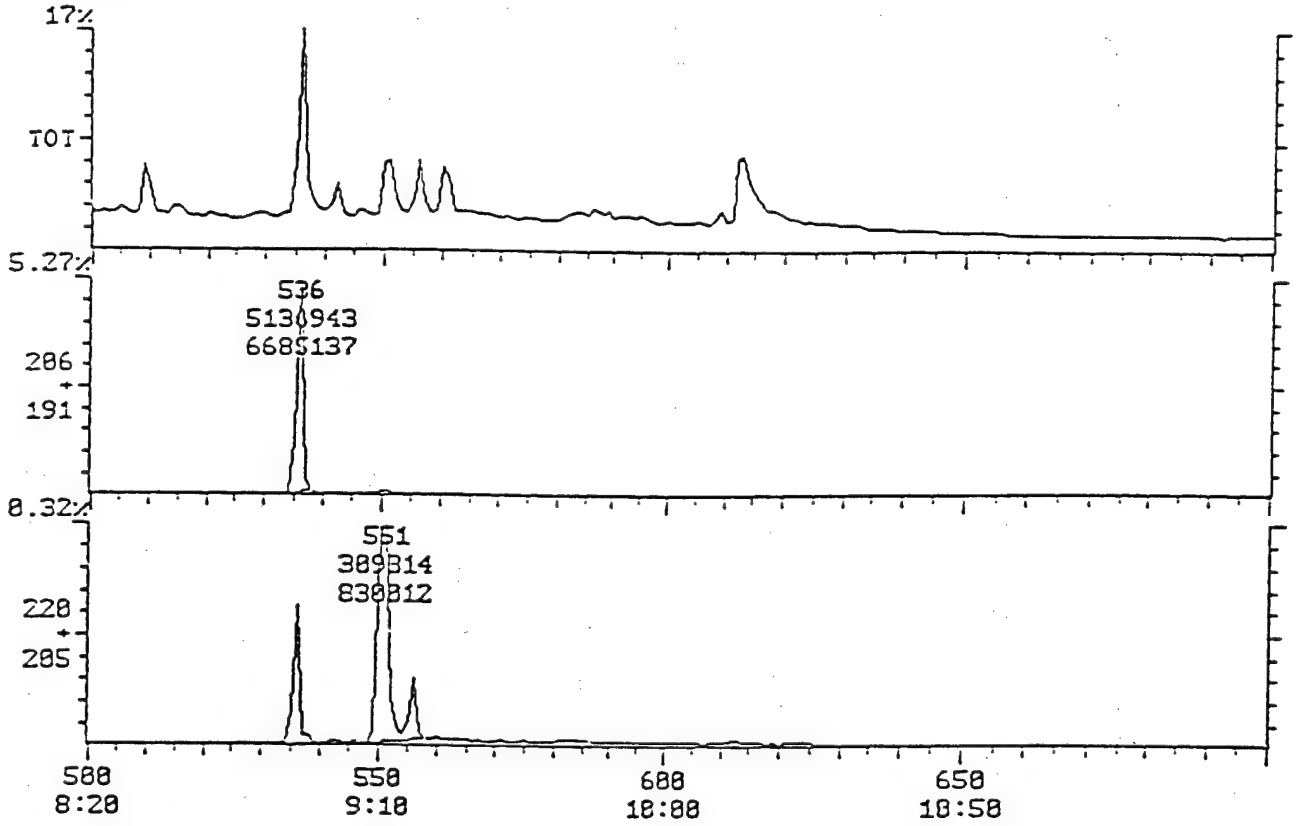
SSBN 738
Engine Room



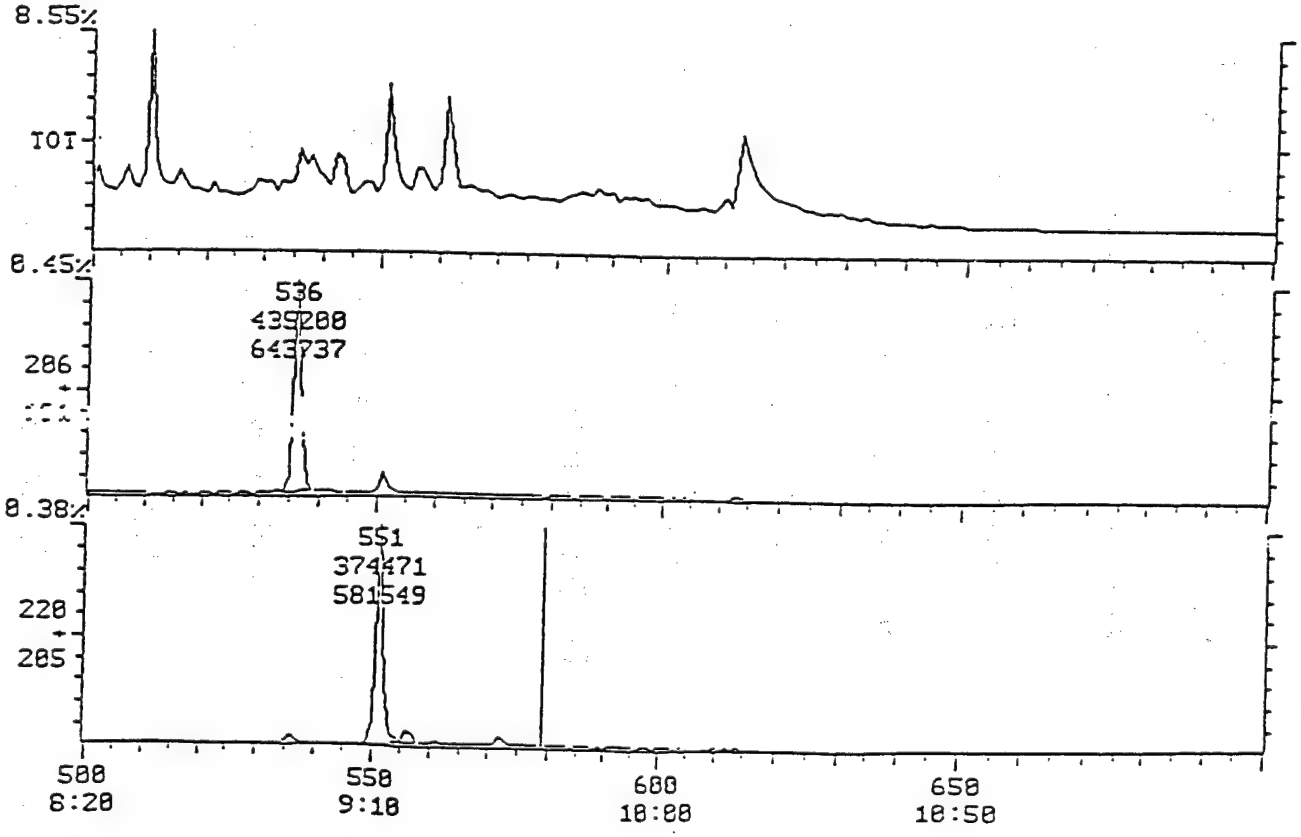
SSBN 738
Near CAMS



SSBN 738
Engine Room



SSBN 738
Near CAMS



Results of Laboratory Experiments

Neither Tenax nor charcoal can sample DBNP when a solution of it is heated

Consistent with literature studies - vapor pressure must be very low

Impinger results

DBP and DBMP can be trapped in CH_2Cl_2, MEK

DBNP can be sampled with MEK only when the solution is heated to 100°C

DBNP can be formed by exposure of DBP to NO_2

10-50 ppm NO_2 + DBP yields DBNP in 1-3 hours

Exposed samples convert completely to DBNP overnight, with no NO_2

Nitration rate may be slightly increased with water

Oil samples exposed to 10-50 ppm NO_2 yield DBNP in 1-2 hours

Low level NO_2 exposure (sub ppm) results in slower reaction rates

Results of Submarine Surface Swipe, Scrapings and Oil Sample Analyses

USS Maryland (SSBN 738)

Yellowed painted surfaces aft showed DBMP and DBNP, but little DBP

Non-yellowed surfaces did not show DBNP or DBMP (aerosols?)

DBP generally absent from surfaces, despite presence in air (reaction)

USS Annapolis (SSN 760)

Yellowed areas showed DBMP and DBNP, with low levels of DBP

Non-yellowed showed no detectable amounts of any phenol

Oil from the vent fog precipitator showed low level of DBNP

SSN 724

Visibly-discolored surface scrapings showed DBMP and DBNP, with no DBP

Other Information

Literature references

German research on nitration of BHT and yellowing of textiles

Swiss work on attempts to sample phenols and nitrophenol from the air

DBNP: "... known to be the cause for various materials turning yellow in the indoor environment ..." - Rothweiler et al., Atmos. Envir., 25, 231 (1991)

LCDR Doug White's comments

Phenols are easily nitrated and, in general, nitrophenols are toxic, absorbed through the skin

Summary

- Significant amounts of DBP and DBMP are airborne (volatilized from the oil)
- DBP, DBMP and DBNP are present in swipes of yellowed surface areas
- It is unlikely that DBNP is airborne
- It is more likely that DBNP is formed by nitration of surface sorbed phenols

Unanswered Questions/Plans

- Continue sampling onboard submarines with and without the yellowing problem
  - What are the sources and levels of NO_2, HNO_3 (and O_3?) in the air?
  - Do aerosols, temperature, humidity or light play a role in nitration?
  - Are there other compounds or reactions leading to surface discoloration?

Appendix B

STUDY OF VITRO AND IN VIVO EFFECTS OF DBNP ON RAT LIVER FATTY ACID BINDING PROTEIN AND RAT LIVER SULFOTRANSFERASES

Final Report for Contract F3360194MT601

Submitted, October 11, 1994

by

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to

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WPAFB Dayton, OH

I. INTRODUCTION

A. Initial observations on DBNP:

In 1992 the Navy Environmental Health Center, Norfolk, VA was made aware of concern about discoloration of bulkheads, bedding, and Naval personnel aboard submarines based at Groton CT. The agent which caused the problem was identified as 2,6-di-t-butyl-4-nitrophenol (DBNP). The yellowing process due to DBNP appeared to arise from reaction of 2,6-di-t-butylphenol, an antioxidant in lubricant 2190 TEP, with NO_2 produced by the action of their on board electrostatic precipitators. Exposure of submariners to DBNP and any potential toxicant action it might have appeared most likely to occur by the oral, inhalation, and dermal routes.

Exploration of the literature (1) showed that DBNP -- originally evaluated for use as a mitocide -- did exhibit substantial mammalian toxicity. This is evidenced by oral and intraperitoneal LD_{50} values for male rats, 270 and 450 mg/kg, respectively. Additional subacute toxicity studies (60 days, daily intraperitoneal injection) and chronic (16 week feeding) studies identified the cumulative DBNP toxicity in the liver, kidney, lung, heart, and spleen. Specific effects deemed pervasive included fatty liver, kidney dysfunction, and cardiovascular problems. A second study (2) produced much elevated liver weights and induction of Phase I and Phase II metabolic pathways. DBNP is also a potent uncoupler of oxidative phosphorylation (3).

It seemed of great interest to ascertain potential bases for toxicant action of DBNP in fatty liver, cardiovascular complications, and endocrinologic alterations. Likely candidates were

deemed to be the fatty acid binding protein of liver (FABP), bile acid, steroid hormone, and catecholamine/phenol sulfotransferases (STs). The first test parameter, FABP, was a likely candidate for examination due to its presumed importance in the biochemistry of lipids. STs were relevant because altering hormone/emulsifier forms via sulfation would affect endocrine responses of the Phase I/II metabolic enzymes. In addition, DBNP seemed likely to interact with FABP and STs due to its structural resemblence to the substrates and inhibitors of these enzyme proteins.

B. DBNP effects on the hepatic fatty acid binding protein:

The fatty acid binding protein (FABP) of rat liver is a 13-15 kdal protein that binds fatty acids and bile acids/salts (4-6). It is believed to be important to transport and metabolism of fatty acids and related lipids (7,8). Also, we have found that FABP can alter the sulfation of hepatotoxic bile (9) acids in vitro and that its tissue levels are altered by numerous lipophilic drugs and endocrine phenomena that disrupt or alter lipid metabolism (10-12). Very recently, its action in diabetes has been suggested (13). Hence, DNBP-tissue interactions could affect the in vivo levels of FABP, and by doing so such FABP alterations could induce fatty liver, cirrhosis and other types of liver disease.

C. DBNP interactions with sulfotransferases:

Sulfotransferase enzymes (STs) of liver catalyze addition of sulfate groups to many hydroxylated biomolecules, including the simple phenols, catecholamines, hormonal steroids, drugs, and

bile salts (see 14, and reviews, 15, 16). They utilize 3'-phosphoadenosine-5'-phosphosulfate (PAPS) as the sulfation coenzyme. Several observations, here, suggest the value of the exploration of DBNP as a sulfation substrate and/or inhibitor. First, p-nitrophenol is a substrate of phenol and catecholamine STs. Second, phenols with bulky substituents (e.g., the polyhalophenols) inhibit phenol STs . Bile acid sulfation will alter the emulsifier capacity of liver and could thus be important to the definition of the lipid content of liver.

II. RATIONALE AND AIMS

It is proposed that some aspects of DBNP action in fatty liver and the other toxicant effects observed may be related to alteration of the FABP and the bile salt/hormone sulfotransferase levels that can be shown by examining a male rat model system in animals from the Naval Medical Research Institute (NMRI) colony, These rats will be used to simplify future comparison with other data obtained by researchers at NMRI. Our aims will be to:

a) examine in vitro effects on FABP and rat liver sulfotransferases that catalyze sulfation of bile salts, catecholamines, and steroid hormones

b) compare effects on these proteins of DBNP perfusion of livers and perfusion with Ringer's solution

c) identify the results of subacute DBNP administration on the parameters identified in a and b.

III. EXPERIMENTAL METHODOLOGY

A. Animals and chemicals:

Male Fischer rats (Charles River, Boston, MA IN) were purchased

at 150-175 g, maintained under controlled conditions at the NMRI and used at 250-400g. All the rats were fed rat chow and water, ad libitum. Rose bengal, enzyme grade sucrose, Trizma (trishydroxymethylaminomethane), KCl, NaCl, and glycolitholithocholate were purchased from Sigma-Aldritch (St. Louis, MO), DEAE-Sephadex A-50, Sephadex G-25, and Sephadex G-75 came from Pharmacia-LKB (Piscataway, NJ), and [^{35}S]-3'-phosphoadenosine-5'-phosphosulfate (PAPS, 2.00 Ci/mmol) were from Dupont-New England Nuclear Inc. (Boston, MA). Unlabeled PAPS was prepared as we have described earlier (17). All of the other reagents and supplies used were of the finest quality available. from standard suppliers.

B. Cytosol, sulfotransferase and initial FABP preparation: Cytosol (18) was prepared after rats were decapitated and livers were quickly removed, trimmed, chilled, and homogenized in 1 mL g^{-1} of 1 C, 0.050 M tris-0.25 M sucrose-3.0 mM 2-mercaptoethanol, pH 7.5 (Buffer 1). Homogenates were centrifuged for 1 h (105,000 x g, Beckman L-565) at 1 C. Then, clear, supernatant cytosol was filtered through 0.2 micron filters and used as the source of FABP and all sulfotransferases (STs).

STs and FABP were in DEAE-Sephadex A-50 fractions. The ion exchange chromatography was performed with cytosol samples from 5 g liver, applied to 1.35 x 23 cm DEAE-Sephadex A-50 columns prepared as we have already described (18). Nearly identical ion exchange chromatograms on two size-paired columns were carried out simultaneously when cytosol samples from experimental and control rats were used. Each chromatogram was developed with a linear gradient that was made up of equal volumes of Buffer 1

and Buffer 1- 500 mM KCl (300 mL each). Liver samples were each applied to the columns nearly simultaneously. The chromatography was followed by examination of A_{280} nm absorbance (protein content) of effluent fractions. Then, measurement of FABP and ST content was carried out as described. Figure 1 shows the FABP and protein content of effluent fractions.

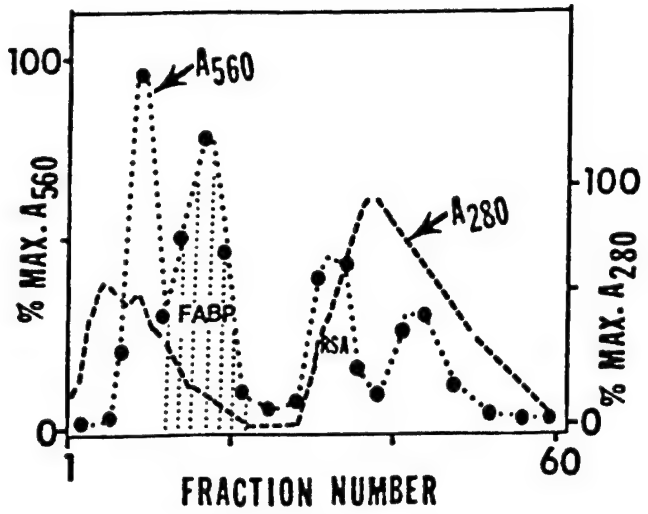

Figure 1 An A-50 chromatogram, showing protein and FABP content

C. FABP quantitation:

This measurement used the rose bengal assay we describe elsewhere (9). Test samples were mixed with ice cold Buffer 1 and 8.00 nmol rose bengal, to yield a volume of 0.700 mL, and incubated (5 min at 4° C). Each reaction mixture was used to quantitate FABP content by a Sephadex G-25 column method or by the equivalent dextran-coated charcoal assay. The two methods were in agreement within 5%. The absorptivity per mg FABP was 1.29.

D. Sulfotransferase assays:

BST assays were carried out by a method we report elsewhere (9, 14). The details are given as an example. Aliquots, 0-0.20 mL of enzyme were mixed with 0.55 ml of 10 μM bile salt in 100 mM KH_2PO_4 (pH 7.00). The sulfation reaction was started with 100 nmol [^{35}S] PAPS (2,000 dpm $nmol^{-1}$) and carried out for 30 min. The reaction was stopped by boiling for 1 min; reaction mixtures were cooled on ice; 2.00 mL of 1.0 M ammonium hydroxide and 5.00

mL of n-butanol were added and mixed for 30 sec. Then, aqueous and butanol layers were separated by centrifugation (5 min, 2000 x g). Samples of butanol layers were counted to identify enzyme levels. Assays of steroid, catecholamine, and phenol sulfotransferase activity we also designed are not described here. The descriptive data are found in our earlier papers (19-21).

E. Statistics:

The statistical significance of the differences between control and experimental groups was determined via Student's test(22).

F. Radioisotopic methods:

The sulfated reaction products were mixed with 5-7 ml of Riafluor scintillation cocktail (Dupont-NEN, Boston MA), depending on the assay method, and counted in an Intertechnique SL-30 scintillation counter, using the channels ratio method (23).

G. FABP isolation:

This process, which we described elsewhere (9), began with the DEAE-Sephadex chromatography cited in III.B. The effluent fractions described were assayed with rose bengal and those which contained FABP were pooled and concentrated to five mL in an Amicon vacuum concentrator, using YM5 membranes. Each concentrated FABP pool was then applied to a 1.95 x 70 cm Sephadex G-75 column, preequilibrated with and eluted with Buffer 1. Again, control and experimental samples were eluted simultaneously from paired columns of the same dimensions. The effluent fractions collected were then assayed for protein and FABP (e.g., Figure 2). The FABP-containing fractions -- highly purified FABP were then pooled and reassayed to identify rat liver FABP content in

mg per g liver or per 100 g bodyweight (BW).

IV. EXPERIMENTAL DESIGN

A. Study of effects of DBNP on hepatic FABP and the ST activity:

Here we will identify the interactions of DBNP with rat liver FABP and with phenol/bile acid/steroid STs, to quickly probe whether the toxicant has any inhibitor potential. Also the ability of DBNPas a phenol ST substrate will be explored.

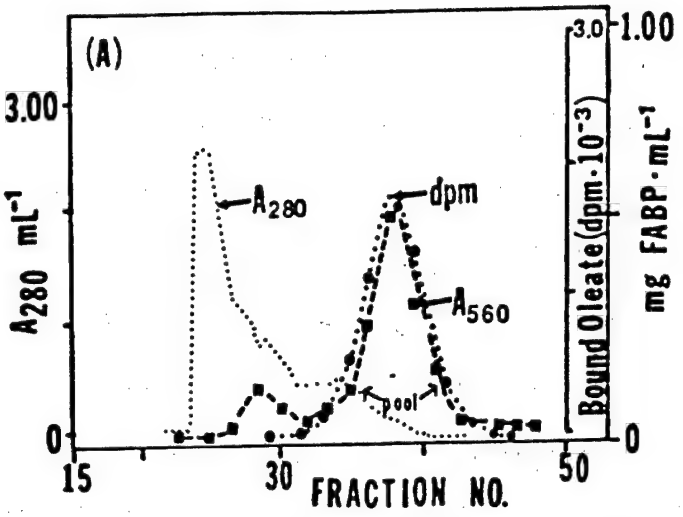

Figure 2 A Sephadex G-75 chromatogram Depicting FABP elution

B. Identify whether DBNP in perfusions alters FABP or ST levels: This study involves examination of liver by cytosol assays and column chromatography described above (on DEAE Sephadex A-50, and Sephadex G-75 columns). The endeavor will quickly probe whether the toxicant has the potential to alter lipid homeostasis by changed FABP or ST levels. It utilize tissue from the standard perfusions in progress at NMRI (25). The perfusions will be carried out at NMRI. The livers will then be placed on ice and taken to my laboratory for immediate processing.

C. Understanding effects of DBNP subacute toxicity on FABP and targeted STs:

This may add a dimension to your ongoing endeavors by enabling preliminary identification of factors associated with fatty liver

and cardiovascular damage already observed (2) The experimental design will be as cited. Rats will be given vehicle or DBNP daily intraperitoneally. At chosen intervals they will be sacrificed and their livers will be perfused for 5 min to bring them to a state similar to that obtained in studies carried out at NMRI. Then, they will be placed on ice and taken to my laboratory for immediate processing.

V. RESULTS

A. In vitro DBNP effects on rat liver FABP, BST, DST, HCST, and EST

In these experiments study of cytosol and DEAE Sephadex A-50 column fractions indicated (3 experiments, not shown) that there were no meaningful differences in BST or DST activity in samples assayed with or without 200 μM DBNP. Similar examination of FABP in Sephadex G-75 column fractions or purified FABP pools from columns (3 experiments, not shown) did not uncover any effects of 200 μM DBNP on FABP activity.

We were unable to carry out a successful examination of DBNP effects on HCST or EST, as the cortisol and estrogen ST assays did not work under any experimental conditions (8 experiments, not shown). This implies that a component of perfusion mixtures used at NMRI prevents the reaction because we carried out successful training assays with untreated liver from rats from our own animal colony.

DBNP was not sulfated by cytosol or ion exchange chromatogram fractions (4 experiments, not shown).

B. Effects of perfusion of control livers with DBNP or Ringer's solution on FABP and STs

1. FABP levels per g liver and per 100 g BW were elevated by DBNP perfusion: Table 1 shows the FABP levels per g and data extrapolated to per 100 g BW in rat livers from males carried through the routine perfusion procedure used at NMRI, with or without 0.36 mM DBNP. Shown is one of two experiments that provided similar data. An average 25% increase of the FABP isolated from the livers perfused with DBNP was obtained.

Table 1 Effect of DBNP perfusion on FABP levels in livers of representative male rats

Exptl Gp	BW(g)	LW(g)	Hepatic FABP in mg FABP per: g liver	100g BW
Control	371	9.46	0.873	3.56
DBNP	372	12.1	1.07	4.34
% FABP Increase			22.6	21.9

2. BST levels per g liver and per 100 g BW were not elevated by DBNP perfusion: Table 2 gives the observed BST levels per g lover and data extrapolated to per 100 g BW in livers carried through the routine perfusion, with or without DBNP. Shown is one of two experiments that provided similar data. A small average difference of under 5% was observed. This was within the expected error of the assay procedure. No differences of individual BST isoenzymes were seen in DEAE Sephadex A-50 chromatography (not shown).

Table 2 Effect of DBNP perfusion on BST levels in livers of representative male rats

Exptl Gp	BW(g)	LW(g)	Hepatic BST in nmol GLCS per: g liver	100g BW
Control	272	9.85	249	904
DBNP	271	11.5	226	944
% BST Change			-9.24	+4.42

3. DST levels were not be obtained, as the cytosol assay was unsuccessful: The DST assay was not successful during the time period in which this experiment series was carried out. We later found that the problem had to do with the long perfusion time and the heparin content of the perfusate. Hence, short perfusions were carried out without heparin in the studies described in Sections 3C1-3.

C. Study of effects of subacute DBNP toxicity on FABP and STs

The rats utilized received daily intraperitoneal injections of 25mg/kg DBNP in dimethy sulfoxide (DBNP group) or the vehicle (controls). They were sacrificed in pairs (1 control + 1 DBNP rat) 33-58 days after the study began. The time period was shorter than planned, due to deaths in the DBNP group from day 30 on.

1. The DBNP-treated rats had enlarged livers Table 3 shows individual body weights, liver weights, and liver to body weight ratios of DBNP and control groups. Note that the body weights of the two groups were similar; the livers of DBNP-treated rats were larger than those of the controls; and liver to BW ratios of the groups differed significantly ($p<0.02$).

Table 3 Body weights(BWs), liver weights (LWs) and LW/BW of rats given DBNP or vehicle

Exptl Gp & No	BW(g)	LW(g)	Lw/BW
Controls(5)	283±16	9.71±0.57	0.0343±0.0011
DBNP (5)	257±17	11.,6±1.3	0.0446±0.0036
t test	-	-	P<0.02

2. FABP levels per g liver but not per 100g BW were decreased significantly by DBNP treatment Table 4 shows the FABP levels in mg FABP per g liver and per 100 g BW in DBNP and Control groups. Data were obtained after cytosol FABP was purified by consecutive DEAE-Sephadex A-50 and Sephadex g-75 chromatography (recall Figures 1 and 21). FABP per g liver decreased significantly by an average of 54% (P<0.2), while the data per 100 g BW showed only a nonsignificant 17% average decrease. The FABP data per 100 g BW (not shown) appeared to be biphasic, with the first three test pairs (days 33-45) differing by an average decrease of of 34% while the last two pairs (days 52-58) differed by a decrease of only 5%.

Table 4 Hepatic FABP levels in rats given DBNP or vehicle

Exptl Gp & No	Hepatic FABP in mg FABP per: g liver	100g BW
Controls(5)	0.743±0.095	4.09±0.57
DBNP (5)	0.341±0.085	3.41±0.74
Avg Effect	-54%	-17%
t test	P<0.02	n.s.

3. Bile salt sulfotransferase(BST) levels per 100 g BW, not per gliver, were increased significantly by DBNP and BSTI appeared to be involved Table 5 shows BST levels (nmol glycolithocholate sulfate, GLCS) made per g liver and per 100 g BW in DBNP or control rats. The data came from assay of BST activity with 4 levels of cytosol. BST activity per g liver did not increase in a statistically significant fashion. The increase of BST activity per 100 g BW averaged 71% (62-84%) and it was statistically significant.

Table 5 Hepatic BST levels in rats given DBNP or vehicle

Exptl Gp & No	Hepatic BST in nmol GLCS per: g liver	100g BW
Controls(5)	231±64	761±198
DBNP (5)	286±78	1300±310
Avg Effect	+24%	-71%
t test	n.s.	P<0.02

Figure 3 shows the results of chromatography of cytosol from a DBNP-treated rat and from a control rat on DEAE Sephadex A-50. As indicated in Methods the two equivalent liver samples were chromatographed side by side on nearly identical ion exchange columns. The data indicate that most of the BST activity in the DBNP-treated rat was due hepatic BST I, the main BST present in female rats(9). The control rat exhibited a typical "male" BST profile that contains lower levels of all three isofunctional BSTs. The BSTs are identified by the salt content of the highest effluent fraction of each enzyme peak. This was one of 3 quite similar experiments.

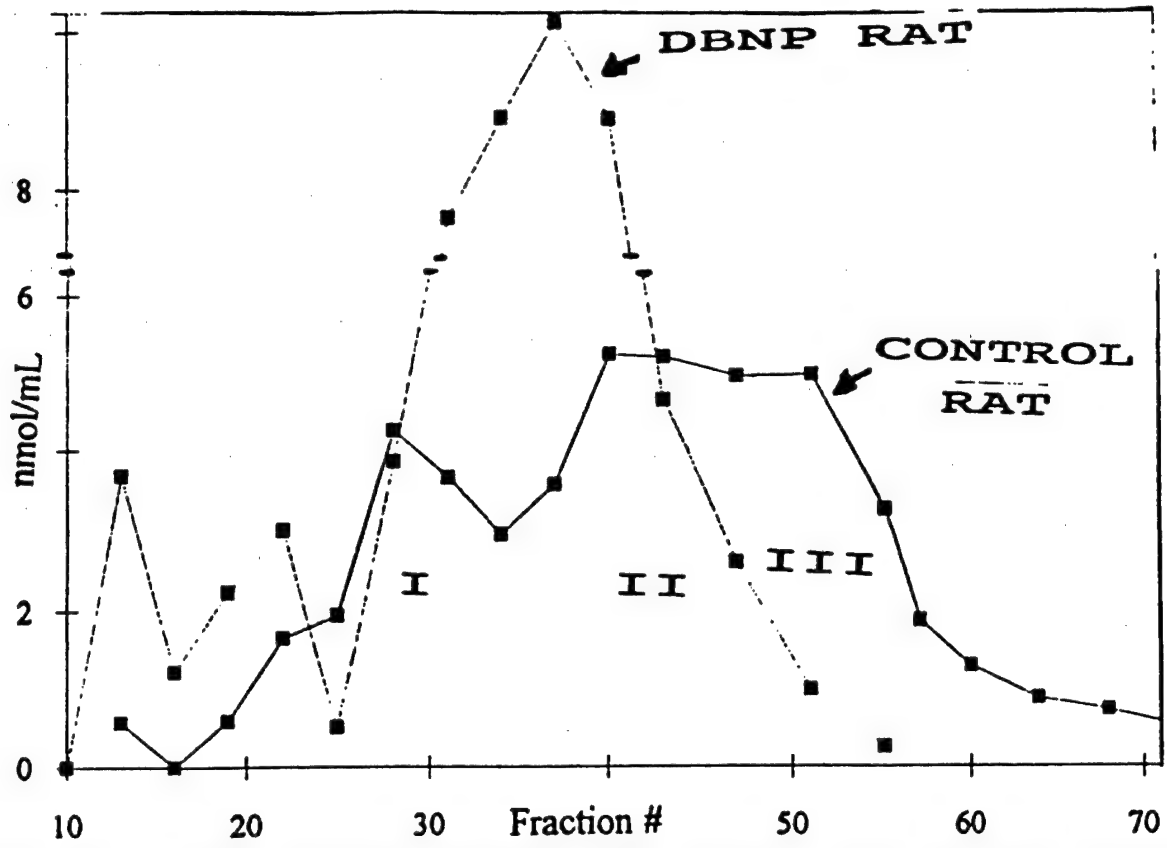

Figure 3. BSTs in DBNP-treated and control rats, an A-50 column

4. Dopamine sulfotransferase(DST) levels per g liver and per 100 g BW, were increased significantly by DBNP and DSTII appeared to be involved Table 6 shows DST levels (nmol dopamine sulfate, DS)per g liver and100 g BW in DBNP or control rats. Data came from assay of DST activity with 4 levels of cytosol. Activity per g liver and 100 g BW increased by an average of 31% and 28% (24-37%), respectively. The increases were statistically significant.

Table 6 Hepatic DST levels in rats given DBNP or vehicle

Exptl Gp & No	Hepatic DST, nmol DS present per: g liver	100g BW
Controls(5)	1530±260	4420±430
DBNP (4)	1170±250	5660±330
Avg Effect	+31%	+28%
t test	P<0.05	P<0.02

Chromatography of cytosol from a DBNP-treated rat and a control rat on DEAE Sephadex A-50 was also carried out(Figure 4). The figure depicts one of four similar experiments. The results indicate that most of the DST activity present in both rats is due to DSTII, the main enzyme we found in both males and females (21). Which enzyme is affected by DBNP administration, here, is presently unclear because of the small overall increase of total DST activity observed (recall Table 6).

Note that the identities of the DSTI and DSTII peaks in the two superimposed ion exchange chromatograms depicted are certain. They were ascertained from the effluent salt content of the peak tubes. There is no ambiguity in the data.

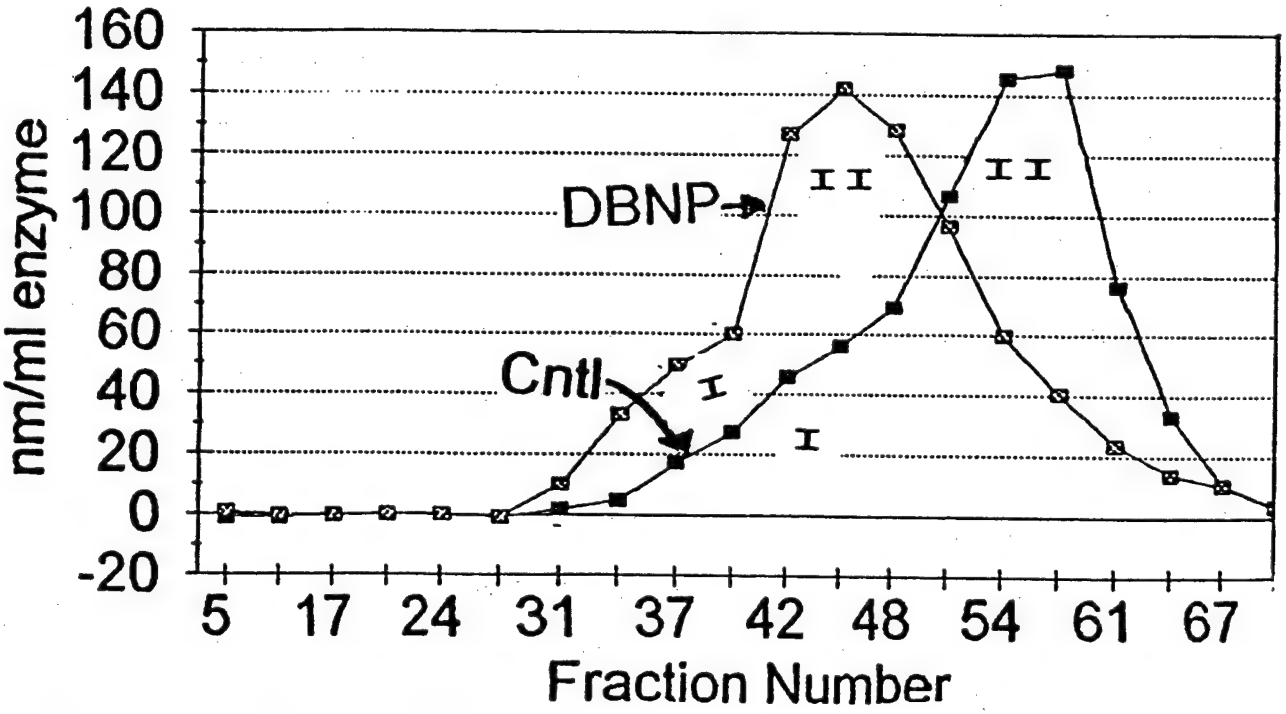

Figure 4. DSTs in DBNP-treated and control rats, as shown by DEAE-Sephadex A-50 chromatograms.

VI. DISCUSSION

The occurrence of DBNP on Naval submarines raises the specter of potential danger to personel that could accrue from this mammalian toxicant (1). The search for clues to such dangers is best begun with examination of parameters identified in animal study. Thus we sought to evaluate potential bases for toxicant action of DBNP in the rat, namely fatty liver and cardiovascular problems. We thus explored rat liver FABP, BST, EST, HCST and DST. FABP was a likely candidate for examination due to its importance in the biochemistry of lipids(7-13). The STs appeared relevant because altering hormone/emulsifier forms via sulfation would affect endocrine responses of the Phase I/II metabolic enzymes. In addition, DBNP seemed likely to interact with FABP and STs due to its structural resemblence to the substrates and inhibitors of these proteins.

Our first studies examined in vitro DBNP effects on FABP and the STs and the potential for DBNP sulfation by DST. We found that DBNP had no effect on FABP or DST and that DBNP was not sulfated either by cytosol or any DEAE Sephadex A-50 chromatograms we used to probe the individual STs. Hence:

1) in vitro interactions between the toxicant and our test parameters are deemed unlikely to obscure study of its biological effects
2) DBNP sulfation is not a probable occurrence in vivo and DBNP sulfate is an unlikely metabolite

Exploration of the effects of perfusion on the test parameters (Table 1) showed a small increase (averaging 25%) of the

isolated FABP and no effect on BST (Table 2) in livers perfused for several hours with 0.36 mM DBNP by the method used to probe other liver parameters at NMRI. Studies of DST and the steroid hormone sulfotransferases aborted due to methodological difficulties associated with the makeup of the perfusant (probably included heparin).

The perfusion data were not conclusive because of time, methodologic considerations and tissue available (only 2 animal sets) in the time frame agreed on. More samples should be looked at, if possible, due to need for resolution of these issues which in the case of DST and FABP seem likely with a few more samples. As to the other STs (HCST and EST), now that the methodologic complications are clear, additional stidies should be both possible and provide clarification.

The study of the effects of subacute DBNP toxicity on FABP and STs was most rewarding. In these rats, which developed enlarged -- presumably -- fatty livers (Table 3). FABP levels per g liver decreased significantly by an average of 54% within 33 days (Table 4). The data per 100 g BW showed what may be a biphasic response, presenting as an average 34% decrease between days 33 and 45 but only 5% between days 52 and 58. As this response could be crucial to development of fatty livers and to identification of long term hepatotoxicity additional animals should be examined. It is also possible that the actual effects were greater than seen because of the fact that the perfusion studies appeared to stabilize FABP. Hence future efforts must factor this observation into experimental plans. It is important

to continue this effort because it may yield valuable insight into understanding the potential diabetogenic complications of FABP raised by others(13) and cirrhotogenic potential that we have suggested (9).

The study of BST activity was also exciting. Though the BST levels did not rise significantly per g liver, a 71% increase per 100 g body weight was observed (Table 5) and this appeared to be due to alteration of relative levels of BSTI (Figure 3), the main BST present in female rats (9). This potential feminization of the BST in liver also supports endocrine alterations in the DBNP-treated rats that makes it important to explore the steroid sulfotransferases with which we were unsuccessful, now that we understand the problems involved. In addition, the BST data are in agreement with our expectations of BST-FABP interrelations (9) this may have cirrhotogenic implications we have suggested and may support the importance of the diminished FABP levels we report here.

Additional support for endocrine alterations arise from our observation of a small but significant elevation of the DST levels per g liver and per 100 g BW in DBNP rats (Table 6). Here, the catecholamine levels in vivo would be altered and lead us to suspect similar and perhaps more extensive alteration of EST and HCST that would modify tissue levels of glucocorticoids and estrogens.It is also important to identify the DST elevated, as DSTII increase would have different results from DSTI elevation (21).

VII. REFERENCES

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